CHARACTERIZATION OF HUMAN SARCOMA ANTIGEN S₃

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Summary.—An antigen common to human sarcomas, S₃, has been further characterized. It is antigenically distinct from human blood-group substances A and B and from heterophile antigens such as Forssman, infectious mononucleosis and serum sickness antigens. Whilst S₃ antigen preparations may contain small amounts of CEA and AFP there is no correlation between S₃ antigen and the presence or amount of these known tumour-associated substances. S₃ antibody can be fully absorbed with guinea-pig kidney but not boiled beef or SRBC. S₃, therefore, is a heterophile substance which has not previously been identified. A seroepidemiological survey confirms that S₃-antibody prevalence is significantly increased in persons with a wide variety of malignant disease, as well as in family members of patients with sarcoma.

We have previously detected (Sethi & Hirshaut, 1976) a tumour-associated antigen common to human sarcomas, using a complement-fixation assay. The prevalence of antibody to this antigen, S₃, is markedly increased in persons with sarcoma, and to a lesser extent in patients with a wide variety of other solid tumours. When a group of patients with sarcoma was followed through surgery and their antibody levels to S₃ determined before and after operation, sharp increases in antibody titres were observed after the removal of the tumour. Titres remained high in those with residual disease but gradually fell to undetectable levels in patients whose malignancy had been completely excised. These initial data (Sethi et al., 1978) suggest that the determination of changes in S₃-antibody levels in sarcoma patients and others with malignancy might be of prognostic value.

This paper reports the results of additional studies to characterize the S₃ antigen. Immunological techniques have been used to determine the heterogeneity of S₃ preparations from different sources, and to discover whether S₃ is related to previously identified antigens in benign and malignant tissues. Seroepidemiological studies have been completed to delineate more fully the distribution of S₃ antibody.

MATERIALS AND METHODS

Cell cultivation.—Human sarcoma cells propagated in tissue culture are the source of S₃ antigen. Details of procedures used for the establishment of sarcoma cell lines have been presented elsewhere (Sethi et al., 1977). Cultured cells were maintained at 37°C in RPMI 1640 medium supplemented with 20% inactivated and filtered foetal calf serum. They were transferred and fed every 2–3 weeks. Cell lines were also checked periodically for mycoplasma by the method of Fogh et al. (1967). Those used to prepare S₃ were established to be free of such contamination.

Preparation of S₃ antigen.—Test antigen was obtained from cultured cells harvested with a rubber policeman. Proteolytic enzymes were not used for cell dispersion. After washing the cells with phosphate-buffered saline (PBS) at pH 7.4, a 20% cell suspension was

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prepared in PBS. Cells were then frozen and thawed x 3 in liquid N2 and a 37°C water bath respectively. Cell membranes were additionally disrupted by sonication (Biosonic IV; VWR Scientific, San Francisco, California) for 2–3 min (10 sec/ml) in 1-min bursts. The crude suspension without further centrifugation or filtration was the antigen source. This material was dispensed for storage into 1-ml polypropylene tubes kept in a freezer at -70°C.

Complement-fixation assay.—In the test for S3, a standard microcomplement fixation method was used (Sever, 1962). All dilutions were made with veronal buffered saline (VBS) pH 7.4. Pooled, titred human cord serum was the complement source. All sera assayed for S3 antibody were stored at -70°C before use. For testing, sera were diluted with an equal volume of VBS, inactivated at 56°C for 30 min, and then checked for anti-complementary activity. Amboceptor (Difeo Laboratories Inc., Detroit, Mich.) was pre-titrated and freshly diluted for incubation with SRBC. Antigen titres were determined by a checkerboard titration, using a standard high-titrated serum from a patient with osteogenic sarcoma as the source of S3 antibody. Each experiment included controls for serum, antigen, complement, and sheep red blood cells (SRBC). The results were recorded on a 0-4+ scale corresponding to the size of the red-cell button and the amount of complement fixed.

To conduct the complement-fixation test, 0.025 ml VBS was delivered to each well in the plate. Serum was then added to the first horizontal row of wells, and serial 2-fold dilutions were made in a vertical direction with 0.025ml diluting loops. Every serum sample was diluted in duplicate using adjacent vertical rows of wells. Then 2 units of antigen were placed in alternate vertical rows of wells and the remaining wells received a similar volume of VBS. Finally, two 100%-haemolytic units of complement were added to all wells, and the plates were incubated overnight in a plastic bag at 4°C. The next day, 0.05ml sensitized SRBC were added to each well. The haemolytic system was a 2% suspension of 4–6-week-old washed SRBC. SRBC were sensitized by incubation with an equal volume of 1:800 diluted rabbit anti-serum against SRBC (amboceptor) at 37°C for 30 min. The whole plate was again incubated at 37°C for 1 h with gentle intermittent shaking. Finally each plate was centrifuged for 5 min at 1000 rev/min and readings were taken. Sera giving a 2+ reaction or greater at a dilution of 1:4 or above, and free of anti-complementary activity, were considered positive for S3 antibody.

Absorption procedures.—Sera to be studied were inactivated at 56°C for 30 min. They were then diluted with VBS to twice their limiting positive dilution. One aliquot remained unabsorbed while other aliquots were mixed with an equal volume of absorbing material. All samples were gently shaken for 3 min and then stored at 4°C overnight. The next day, after centrifugation at 2000 rev/min for 15 min, supernatants were removed by pipette and tested against a standard S3 preparation in a complement-fixation assay. Unabsorbed serum was run as a control with each assay. Absorbs included S3 antigen preparation, boiled beef (BB), guinea-pig kidney (GPK), sheep red blood cells (SRBC), Type A and B RBC and carcinoembryonic antigen (CEA).

Other assays.—CEA and α-fetoprotein (AFP) levels were measured by radioimmunoassay (RIA) using techniques previously described (Hansen et al., 1971; Waldman & McIntire, 1972).

These tests were performed respectively in the laboratories of Drs Morton Schwartz and Thomas Waldmann. Anti-Epstein–Barr Virus (EBV) viral capsid antigen (VCA) titres were performed by the method of Hirshaut et al. (1969).

RESULTS

The cell lines used for S3 preparations are presented in Table I. To compare S3

| Diagnosis          | Patient | Cell line | S3 antigen |
|--------------------|---------|-----------|------------|
| Osteosarcoma       | WR      | ROS-23    | -          |
| Schwannoma         | CR      | RSS-25    | -          |
| Liposarcoma        | JB      | BLS-50    | -          |
| Rhabdomyosarcoma   | GK      | KRS-70    | +          |
| Chondrosarcoma     | MB      | BCS-72    | +          |
| Fibrosarcoma       | JR      | RFS-75    | +          |
| Leiomyosarcoma     | JL      | LMS-76    | +          |
| Liposarcoma        | SH      | HLS-80    | +          |
| Synovial sarcoma   | CH      | CS-145    | +          |
| Spindle-cell sarcoma| ML | LSCS-147  | +          |
| Osteosarcoma       | PR      | ROS-151   | -          |
| Liposarcoma        | FM      | MLS-164   | +          |
antigen preparation from 3 sources, 6 $S_3^+$ and 5 $S_3^-$ sera were tested against antigen derived from cell lines BCS-72, LMS-76, RFS-75. As seen in Table II, each of the sera showed an identical reaction against the 3 different $S_3$ preparations. However, titres against $S_3$ from BCS-72 tended to be one 2-fold dilution lower. In a cross-absorption experiment, using $S_3$ antigen prepared from cell lines CS-145 and LSCS-147 (Table III) allreactivity of positive antisera to the $S_3$ antigen preparations was removed after absorption with either antigen. Absorptions were conducted at twice the limiting dilution of each test serum with no reactivity remain-

### Table II. — Cross-reactions (titres) against $S_3$ preparations

| Antigen preparations | BCS-72 | LMS-76 | RFS-75 |
|-----------------------|-------|-------|-------|
| Serum donor           |       |       |       |
| Osteosarcoma          | 1/16  | 1/32  | 1/32  |
| Neurogenic sarcoma     | 1/32  | 1/64  | 1/64  |
| Myxoliposarcoma       |       |       |       |
| Fibrous histiocytoma   | 1/32  | 1/64  | 1/64  |
| Liposarcoma            |       |       |       |
| Fibrosarcoma           | 1/32  | 1/64  | 1/64  |
| Myxoliposarcoma        | 1/32  | 1/64  | 1/64  |
| Rhabdomyosarcoma       | 1/32  | 1/64  | 1/64  |
| Myxoliposarcoma        |       |       |       |
| Liposarcoma            |       |       |       |
| Liposarcoma            |       |       |       |

The association of $S_3$ with non-tumour-associated blood groups or heterophile antigens was studied by absorbing 8 $S_3^+$ and 4 $S_3^-$ sera with SRBC, GPK, BB and human blood cells of Types A and B. The data obtained are presented in Table V. None of the reactivity of positive sera was absorbed with SRBC, BB and Type A and B human RBC. All the activity, however, was removed from the same sera after absorption with GPK.

$S_3$ was distinguished from CEA by determination of CEA levels in $S_3^+$ and $S_3^-$ sera and in $S_3^+$ and $S_3^-$ sera. Small quantities of CEA were present in 4 of the 5 $S_3$ antigen preparations tested. There was no correlation, however, as evident in Table VI, between the presence of CEA and $S_3$ reactivity. Both $S_3^+$ and $S_3^-$ sera were found to contain measurable CEA (Table VI). Absorption of 2 $S_3$ antibody$^+$ sera with purified CEA caused no loss of anti-$S_3$ reactivity.

AFP levels in $S_3^+$ antigen preparations and antisera were also assayed. Results are shown in Table VII. Again low levels of AFP were found in antigen and anti-

### Table III. — Cross-absorption with $S_3$ preparations

| Titres | Cross absorption |
|--------|------------------|
|        | CS: LSCS-145     | CS: LSCS-147 |
| Serum donor |        |        |
| Osteosarcoma | 1:64 | 1:64 | -/+ -/+ |
| Spindle-cell sarcoma | 1:32 | 1:32 | -/+ -/+ |
| Normal |      |      |        |

* Post/Pre. Sera absorbed at twice limiting dilution.

### Table IV. — Summary of absorptions for anti-$S_3$ specificity with $S_3^+$ and $S_3^-$ antigen preparations

| Serum donor | Anti-$S_3$ titres on absorption | Anti-EBV-VCA titres on absorption |
|-------------|---------------------------------|----------------------------------|
|             | Un-absorbed (S$_3^-$)          | (S$_3^+$)                        |
|             | ROS-151                         | MLS-164                         |
|             | LSCS-147                        |                                 |
|             | (S$_3^+$)                       |                                 |
| Osteosarcoma| 1:64                            |                                 |
| Liposarcoma | 1:32                            |                                 |
| Osteosarcoma| 1:8                             | N.D.                            |

| Serum donor | Anti-$S_3$ titres on absorption | Anti-EBV-VCA titres on absorption |
|-------------|---------------------------------|----------------------------------|
|             | Un-absorbed (S$_3^-$)          | (S$_3^+$)                        |
|             | ROS-151                         | MLS-164                         |
|             | LSCS-147                        |                                 |
|             | (S$_3^+$)                       |                                 |
| Osteosarcoma| 1:32                            |                                 |
| Liposarcoma | 1:32                            |                                 |
| Osteosarcoma| 1:8                             | N.D.                            |
Fibrosarcoma
Myxoliposarcoma
Rhabdomyosarcoma
Neurogenic sarcoma
Liposarcoma
Rhabdosarcoma
Osteosarcoma
Lymphoproliferative neoplasms

264 found in antibody.

Histiocytoma
dilution.

Myxoliposarcoma
Fibrosarcoma
Liposarcoma
Rhabdomyosarcoma
Liposarcoma
Liposarcoma

antibody.

to those with a variety of carcinomas and to a lesser degree in persons with lymphoproliferative neoplasms. Only 4 of the 54 normal subjects tested (7%) were found to have circulating levels of antibody to S₃, whereas sarcoma family members have higher prevalence (40%).

**Table V.—Summary of S₃ absorption studies**

| Serum donor | Anti-S₃ activity | Absorption material† |
|-------------|-----------------|----------------------|
| Rhabdomyosarcoma | + | GPK -/+ | BB +/+ | SRBC +/+ | A +/+ | B +/+ | S₃ -/+ |
| Fibrosarcoma | - | GPK -/- | BB -/- | SRBC -/- | A -/- | B -/- | S₃ -/- |
| Osteosarcoma | + | GPK +/- | BB +/+ | SRBC +/+ | A +/+ | B +/+ | S₃ N.T. |
| Liposarcoma | + | GPK +/+ | BB +/+ | SRBC +/+ | A +/+ | B +/+ | S₃ N.T. |
| Osteosarcoma | + | GPK +/+ | BB +/+ | SRBC +/+ | A +/+ | B +/+ | S₃ N.T. |
| Fibrosarcoma | + | GPK +/+ | BB +/+ | SRBC +/+ | A +/+ | B +/+ | S₃ N.T. |
| Neurogenic sarcoma | + | GPK +/+ | BB +/+ | SRBC N.T. | A N.T. | B N.T. | S₃ -/+ |
| Myxoliposarcoma | + | GPK +/+ | BB +/+ | SRBC N.T. | A N.T. | B N.T. | S₃ -/+ |
| Rhabdomyosarcoma | + | GPK +/+ | BB +/+ | SRBC N.T. | A N.T. | B N.T. | S₃ -/+ |
| Myxoliposarcoma | + | GPK +/+ | BB +/+ | SRBC N.T. | A N.T. | B N.T. | S₃ -/+ |
| Liposarcoma | - | GPK -/- | BB -/- | SRBC -/- | A -/- | B -/- | S₃ -/- |
| Liposarcoma | - | GPK -/- | BB -/- | SRBC -/- | A -/- | B -/- | S₃ -/- |

* Post/Pre.
† Absorptions at twice limiting dilution.

Body samples but there was no correlation between AFP levels and quantity of S₃ antigen or antibody in the same material.

To confirm previous observations about the seroepidemiology of S₃ antibody, the number of patients studied has been increased, and now includes 378 with a variety of malignancies and 54 controls (Table VIII). Patients with sarcoma cont-

**Table VI.—CEA analysis of S₃ preparations and sera from sarcoma patients**

| Antigen preparation | S₃ antigen | CEA (ng/ml) |
|---------------------|------------|-------------|
| ROS-23 | - | 0-0 |
| RSS-25 | - | 10-0 |
| BLS-50 | - | 0-0 |
| KRS-70 | + | 1-0 |
| BCS-72 | + | 17-0 |
| Serum donor | Anti-S₃ titre | CEA (ng/ml) |
| Liposarcoma | - | 7-0 |
| Liposarcoma | - | 11-0 |
| Rhabdomyosarcoma | 1:64 | 12-0 |
| Liposarcoma | 1:64 | 10-0 |
| Serum donor | Absorption with CEA |
| Fibrosarcoma | +/+ * |
| Myxoliposarcoma | +/+ |
| Histiocytoma | -/- |

* Post/Pre. Sera absorbed at twice limiting dilution.

**Table VII.—AFP analysis of S₃ preparations and sera from sarcoma patients**

| Antigen preparation | S₃ antigen | AFP (ng/ml) |
|---------------------|------------|-------------|
| ROS-23 | - | 21-0 |
| RSS-25 | - | 35-0 |
| BLS-50 | - | 14-0 |
| KRS-70 | + | 19-5 |
| BCS-72 | + | 52-0 |
| HLS-80 | + | 29-0 |
| Serum donor | Anti-S₃ titre | AFP (ng/ml) |
| Liposarcoma | - | 19-0 |
| Liposarcoma | - | 20-0 |
| Rhabdomyosarcoma | 1:64 | 14-0 |
| Liposarcoma | 1:64 | 14-0 |

**Table VIII.—Distribution of S₃ antibody in patients with malignant neoplasm**

| Diagnosis | Tested | + | % |
|-----------|--------|---|---|
| Sarcoma | 43 | 32 | 74 |
| Sarcoma family members | 20 | 8 | 40 |
| Carcinoma | | | |
| Breast | 28 | 15 | 53 |
| Head and neck | 25 | 13 | 52 |
| Lung | 27 | 14 | 52 |
| Colon | 30 | 14 | 47 |
| Ovary | 31 | 13 | 42 |
| Melanoma | 29 | 12 | 41 |
| Prostate | 22 | 7 | 32 |
| Lymphoproliferative malignancies | | | |
| Lymphosarcoma | 29 | 11 | 38 |
| Reticulum-cell sarcoma | 27 | 9 | 33 |
| Hodgkin’s disease | 32 | 10 | 31 |
| Acute lymphocytic leukaemia | 19 | 4 | 21 |
| Normal | 54 | 4 | 7 |
DISCUSSION

Characterization of S₃ requires preparation of large quantities of antigen. Unfortunately, it is difficult to predict whether a given passage of a human sarcoma cell line will contain detectable S₃ (Sethi & Hirshaut, 1978). This places practical limits on the size of any single culture passage prepared from different passages of the same lines and from different lines. It is therefore particularly important that S₃ antigen prepared from several cell lines has been found to be immunologically identical.

The complete absorption of S₃ antibody from test sera by GPK, but not SRBC or BB, establishes S₃ as a new heterophile antigen distinct from the well-known heterophile substances associated with SRBC, such as Forssman, infectious mononucleosis and serum-sickness antigens. The availability of a second source of S₃, GPK, will also be of assistance in characterization studies. This may be particularly important for biochemical isolation which requires large volumes of starting materials.

Heterophile antigens related to SRBC (particularly Forssman antigen) may be expressed more frequently by malignant than normal tissues (Milgrom et al., 1973). Increased heterophile antibody titres have been reported in patients with Hodgkin's disease (Kasukawa et al., 1976), sarcoma (Feit et al., 1977) and other neoplastic diseases (Southam et al., 1951). They are decreased in carcinoma of the lung (Kitamura et al., 1979). Another sarcoma antigen discovered in this laboratory, S₁ (Hirshaut et al., 1974), has also recently been shown to be a heterophile substance not found on SRBC. S₁ antibody prevalence is consistently higher in patients with malignancy. We have now identified S₃ antibody as another heterophile antibody the prevalence of which is increased in cancer patients.

The presence of CEA and AFP in some S₃ antigen preparations and S₃ antisera makes it mandatory to distinguish these from S₃. The lack of any relationship between the presence of these two well-characterized tumour antigens and S₃ antigen or antibody titres makes it unlikely that there is any cross-reactivity. For CEA this is confirmed by the inability of purified CEA to absorb S₃ antibody.

While the highest prevalence of S₃ antibody has so far been found in patients with sarcoma, the expanded seroepidemiologic investigation confirms that patients with a wide variety of malignancies have an increased prevalence of antibody to S₃, as do family members of those with sarcoma. This lack of specificity precludes the use of an S₃ assay for diagnosis. However, the increased prevalence of antibody in persons with so many different neoplastic diseases implies that changes in S₃ antibody levels may be of prognostic value in patients with a variety of malignant conditions.

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