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 $\times 3$ in liquid N_2 and a $37^\circ C$ 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 $-70^\circ C$.

Complement-fixation assay.—In the test for S_3 , 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 S_3 antibody were stored at $-70^\circ C$ before use. For testing, sera were diluted with an equal volume of VBS, inactivated at $56^\circ C$ for 30 min, and then checked for anti-complementary activity. Amboceptor (Difco Laboratories Inc., Detroit, Mich.) was pre-titred and freshly diluted for incubation with SRBC. Antigen titres were determined by a checkerboard titration, using a standard high-titred serum from a patient with osteogenic sarcoma as the source of S_3 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 $4^\circ C$. 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 $37^\circ C$ for 30 min. The whole plate was again incubated at $37^\circ C$ 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 S_3 antibody.

Absorption procedures.—Sera to be studied were inactivated at $56^\circ C$ 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 $4^\circ C$ overnight. The next day, after centrifugation at 2000 rev/min for 15 min, supernatants were removed by pipette and tested against a standard S_3 preparation in a complement-fixation assay. Unabsorbed serum was run as a control with each assay. Absorbants included S_3 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 S_3 preparations are presented in Table I. To compare S_3

TABLE I.—*Cell lines used to prepare S_3 antigen*

Diagnosis	Patient	Cell line	S_3 antigen
Osteosarcoma	WR	ROS-23	—
Schwanoma	CR	RSS-25	—
Liposarcoma	JB	BLS-50	—
Rhabdosarcoma	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	+

TABLE II.—*Cross-reactions (titres) against S₃ preparations*

Serum donor	Antigen preparations		
	BCS-72	LMS-76	RFS-75
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	—	—	—

antigen preparation from 3 sources, 6 S₃⁺ and 5 S₃[−] 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 S₃ preparations. However, titres against S₃ from BCS-72 tended to be one 2-fold dilution lower. In a cross-absorption experiment, using S₃ antigen prepared from cell lines CS-145 and LSCS-147 (Table III) all reactivity of positive antisera to the 2 S₃ 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 III.—*Cross-absorption with S₃ preparations*

Serum donor	Titres		Cross absorption	
	CS-145	LSCS-147	CS-145	LSCS-147
	CS-145	LSCS-147	CS-145	LSCS-147
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-S₃ specificity with S₃⁺ and S₃[−] antigen preparations*

Serum donor	Anti-S ₃ titres on absorption				Anti-EBV-VCA titres on absorption			
	Un-absorbed	ROS-151 (S ₃ [−])	MLS-164 (S ₃ ⁺)	LSCS-147 (S ₃ ⁺)	Un-absorbed	ROS-151 (S ₃ [−])	MLS-164 (S ₃ ⁺)	LSCS-147 (S ₃ ⁺)
Osteosarcoma	1:64	1:64	—	—	1:320	1:640	1:320	1:320
Liposarcoma	1:32	1:32	—	—	1:320	1:320	1:320	1:320
Osteosarcoma	1:8	1:8	—	N.D.	N.D.	N.D.	N.D.	N.D.

ing after absorption. The results showed the 2 antigens to be fully cross-reactive.

To confirm the anti-S₃ specificity of the absorptions, serum samples before and after absorption with an S₃[−] and 2 S₃⁺ preparations were titred for anti-S₃ and anti-EBV-VCA levels. The data are summarized in Table IV. ROS-151, an S₃[−] preparation, did not absorb any anti-S₃ activity from the sera, but MLS-164 and LSCS-147 absorbed anti-S₃ activity completely. No changes in the anti-EBV-VCA titres were seen when the absorptions were conducted with either S₃⁺ or the S₃[−] preparation.

The association of S₃ with non-tumour-associated blood groups or heterophile antigens was studied by absorbing 8 S₃⁺ and 4 S₃[−] 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.

S₃ was distinguished from CEA by determination of CEA levels in +ve and −ve antigen preparations and in +ve and −ve sera. Small quantities of CEA were present in 4 of the 5 S₃ antigen preparations tested. There was no correlation, however, as evident in Table VI, between the presence of CEA and S₃ reactivity. Both S₃⁺ and S₃[−] sera were found to contain measurable CEA (Table VI). Absorption of 2 S₃ antibody⁺ sera with purified CEA caused no loss of anti-S₃ reactivity.

AFP levels in S₃⁺ 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 V.—*Summary of S₃ absorption studies*

Serum donor	Anti-S ₃ activity	Absorption material†					
		GPK	BB	SRBC	A	B	S ₃
Rhabdomyosarcoma	+	-/+*	+/+	+/+	+/+	+/+	-/+
Fibrosarcoma	—	-/-	-/-	-/-	-/-	-/-	-/-
Osteosarcoma	+	-/+	+/+	+/+	+/+	+/+	N.T.
Liposarcoma	+	-/+	+/+	+/+	+/+	+/+	-/+
Osteosarcoma	+	-/+	+/+	+/+	+/+	+/+	N.T.
Fibrosarcoma	+	-/+	+/+	+/+	+/+	+/+	-/+
Neurogenic sarcoma	+	-/+	+/+	+/+	N.T.	N.T.	-/+
Myxoliposarcoma	+	-/+	+/+	+/+	N.T.	N.T.	-/+
Rhabdosarcoma	+	-/+	+/+	+/+	N.T.	N.T.	-/+
Myxoliposarcoma	—	-/-	-/-	-/-	N.T.	N.T.	-/-
Liposarcoma	—	-/-	-/-	-/-	N.T.	N.T.	-/-
Liposarcoma	—	-/-	-/-	-/-	N.T.	N.T.	-/-

* 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 con-

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

Antigen preparation	S ₃ 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-S ₃ titres	AFP (ng/ml)
Liposarcoma	—	19.0
Liposarcoma	—	20.0
Rhabdomyosarcoma	1:64	14.0
Liposarcoma	1:64	14.0

Antigen preparation	S ₃ antigen	CEA (ng/ml)
ROS-23	—	1.0
RSS-25	—	10.0
BLS-50	—	0.0
KRS-70	+	1.0
BCS-72	+	17.0

Serum donor	Anti-S ₃ titre	CEA (ng/ml)
Liposarcoma	—	7.0
Liposarcoma	—	11.0
Rhabdomyosarcoma	1:64	12.0
Liposarcoma	1:64	19.0

Serum donor	Absorption with CEA
Fibrosarcoma	+/+*
Myxoliposarcoma	+/+
Histiocytoma	-/-

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

tinue to have the highest prevalence of S₃ antibody. Increased prevalence is also found in those with a variety of carcinomas and to a lesser degree in persons with lymphoproliferative neoplasms. Only 4 of

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 malignant diseases 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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