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A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes

Staffan Paulie[1], Barbro Ehlin-Henriksson[2], Håkan Mellstedt[3], Hannu Koho[1], Hedi Ben-Aissa[1] and Peter Perlmann[1]

[1] Department of Immunology, University of Stockholm, S-10691 Stockholm, Sweden
[2] Department of Tumor Biology, Karolinska Institute, S-10401 Stockholm, Sweden
[3] Radiumhemmet, Karolinska Hospital, S-10401 Stockholm, Sweden

Summary. We have previously described the derivation of a monoclonal antibody, S2C6, to a novel 50 Kdalton antigen associated with human urinary bladder carcinoma. No reactions were obtained with carcinomas of unrelated origin or with normal urothelial cells. However, the antibody also reacted with a similar antigen on some cell lines of B lymphocyte origin. Using large panels of target cells we have now shown that this reactivity was entirely restricted to cells of the B lineage within the haematopoietic system. As opposed to its apparent restriction to malignant cells of the urothelium, the S2C6 antigen was expressed by normal B lymphocytes as well as by many malignant B cells (chronic lymphocytic leukaemia, hairy cell leukaemia and immunocytoma). Pre-B cells derived from acute lymphocytic leukaemia and plasma cells from multiple myeloma lacked the antigen. Expression was significantly enhanced on cultured B cells from Burkitt lymphomas and on Epstein-Barr virus-transformed lymphoblastoid cell lines including those of the pre-B phenotype derived from fetal bone marrow. As judged from the molecular size and the distribution pattern displayed by the S2C6 antigen it appears to be distinct from other B cell antigens previously described. A possible relation of the S2C6 antigen to a receptor for B cell growth factors is discussed.

Introduction

The pursuit of defining antigens associated with human tumours has resulted in the identification of a growing number of such tumour marker molecules [for reviews see 6 and 23]. Some of these have proven to be of potential clinical use [13, 24] while the applicability of others remains to be established. A common feature for most of these antigens is that they are quantitatively more highly expressed by a certain type or group of tumours while they are lacking or are found in significantly lower amounts in normal tissue or non-related tumours. In a few cases, however, antigens selectively associated with one type of tumour have been shown to be present in comparable amounts on a completely different cell type, without apparent restriction to malignancy. This is the case with an osteosarcoma-associated antigen which is also expressed on mitogen-stimulated blood mononuclear cells [22]. Similarly, we have recently described a monoclonal antibody (S2C6) detecting a surface membrane located antigen of 50 Kdaltons (p50) present on transitional cell carcinomas of the urinary bladder as well as on cell lines of B lymphocyte origin and on a subpopulation of normal blood lymphocytes [11, 20]. In the present study we have extended our investigations on the reactivity of this antibody to cells of the haematopoietic system. This was done by testing a large panel of normal or malignant cells, derived from freshly isolated blood or various tissues as well as from established cell lines of haematopoietic origin.

Offprint requests to: S. Paulie

Material and methods

Normal blood cells. Cells from peripheral blood were fractionated to obtain enriched populations of granulocytes, monocytes, lymphocytes (PBL), erythrocytes and platelets. *Lymphocytes* were purified by gelatin sedimentation, treatment with carbonyl iron and centrifugation on Ficoll-Isopaque (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by Perlmann et al. [21]. Lymphocytes obtained by this procedure contained $<10\%$ of non-lymphocytic cells. To obtain *monocytes*, cells from the gelatin supernatant were washed once in TH-FCS (Tris-buffered Hank's salt solution with 2% fetal calf serum). They were then suspended in 50% FCS to a cell concentration of approximately 10^7 cells/ml then 5 ml of this suspension was added to 25 cm^2 tissue culture flasks (Falcon Plastics, Oxnard, Calif. USA) and the cells were left to adhere for 60 min at 37 °C. Non-adherent cells were removed by washing gently and the adherent cells were loosened by overnight incubation at 4 °C in TH with low serum content (2% FCS). The cells were more than 70% pure monocytes as judged by morphological appearence after staining with May-Grünwald-Giemsa. *Granulocytes* tend to selectively sediment together with the erythrocytes in the gelatin sedimentation step and this fraction was thus exploited for the purification of these cells. By resuspending the sedimented material once more in 3% gelatin the supernatant formed after 20 min sedimentation was collected. After washing in TH it was used as the granulocyte fraction containing 60%–80% granulocytes. *Erythrocytes* for testing were obtained from the sediment. *Platelets* were derived from heparinized blood by removal of other cells by centrifugation at 500 g. The supernatant was collected and the platelets were washed twice in TH-FCS before use.

E-rosetting. The purified lymphocytes were further fractionated according to their ability to form rosettes with

sheep red blood cells [8]. Briefly, lymphocytes were mixed with neuraminidase-treated sheep erythrocytes and after overnight incubation at 4 °C, rosette forming cells were separated from the E^- cells by centrifugation on Ficoll-Isopaque. The T cell enriched E^+ fraction was obtained from the pellet while the B and null cell enriched E^- subpopulation was retrieved from the interphase. Contaminating erythrocytes in the E^+ fraction were lysed with distilled water.

Tissue cells. Cell suspensions from tonsil, spleen and thymus were obtained by mincing the tissue with scissors and passage through a fine metal net. Larger clumps were left to sediment for 1 min and the supernatant was collected and washed twice in TH-FCS. Over 98% of cells obtained by this procedure were viable and mostly in the form of single cells.

Heterologous cells. Lymphoid cells from four different species were also investigated for the expression of the S2C6 antigen. Spleen cells from rat and rabbit were obtained in the same way as described for human tissue. Heparinized blood from horse and monkey was purified to obtain PBL using the same conditions as described above.

Malignant cells. The lymphoid cells of 33 patients with various B lymphoproliferative malignancies were studied. These included 3 patients with non-T common acute lymphoid leukaemia (ALL), 13 patients with B-type chronic lymphocytic leukaemia (B-CLL), 4 with hairy cell leukaemia (HCL), 6 with non-secretory immunocytoma (IC), 5 with immunoglobulin (Ig) secreting immunocytoma (IC-s) and 2 cases of multiple myeloma. Also tested were 7 malignancies of non-B origin. These were 3 cases of T-type chronic lymphocytic leukaemia (T-CLL), 3 with chronic myeloid leukaemia (CML) and one case of acute non-lymphoid leukaemia (ANLL). The Kiel nomenclature was used for the histopathological classification of the non-Hodgkin lymphomas [14]. Except for the two myelomas which were obtained from bone marrow, all cells were purified from whole blood according to the procedure described for PBL.

Cell lines. A total of 24 cell lines of Burkitt lymphoma (BL) origin, 4 cell lines derived from T cell malignancies and 1 histiocytic lymphoma line were used in the tests (Fig. 1; for references see 2). Other cells included 2 subcultures from an Epstein-Barr virus (EBV)-genome positive pre-B cell lymphoma and EBV-transformed lymphoblastoid lines from peripheral blood and from fetal bone marrow (Ernberg et al. in prep.). All cells were cultured in RPMI 1640 (Gibco Biocult Labs., Ltd., Glasgow, Scotland) supplemented with 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml) and 5% FCS (or 10% new born calf serum).

Antibodies. All antibodies used were monoclonal and of mouse origin. They were used either in the form of culture supernatant, ascites or purified immunoglobulin. Antibody S2C6 (IgG1) was derived from an immunization with bladder carcinoma cells [11]. Antibodies directed to framework epitopes of human HLA-DR [7] antigens were a gift from Dr. S. Ferrone. Antibodies to the HLA-DC associated Leu-10 antigen were purchased (Beckton Dickinson, Mechelen, Belgium) as were antibodies to the B lymphocyte-associated antigens B1 and B2 (Coulter Electronics Inc., Hialeah, Ffa., USA). Other antibodies used were the J5 antibody against the CALLA antigen, OKM1 directed against blood monocytes and granulocytes, the pan-T antibody OKT 3 (all from Ortho- Raritan, NJ, USA), antibodies to the plasma cell antigens PC-1 and PCA-1 (a gift from Dr. S. F. Schlossman) and antibodies to the human kappa and lambda light chains (a gift from Dr. N. R. Ling).

Fig. 1. Reactivity of the S2C6 antibody with cell lines of haematopoietic origin

Indirect immunofluorescence. Indirect immunofluorescence (IFL) was performed using either cells briefly fixed with formaldehyde on multitest slides (Novakemi AB, Stockholm, Sweden) or cells in suspension without prior fixation. Results from parallel tests using cells with or without prior fixation gave comparable numbers of positive cells as well as a similar staining intensity for the antibodies used. The test procedures for both methods were similar and have been described in detail elsewhere [11]. Briefly, the cells were incubated with antibody for 30 min at 4 °C, using culture medium and a monoclonal antibody to human growth hormone (IgG 1; gift of Dr. I. Jonsdottir) as controls for background staining. After washing, the cells

Table 1. Reactivity of S2C6 antibody with various blood cells. Comparison with other surface markers

		Fluorescence (%)					
Antibody	Exp.	RBC/platelets	Granulocytes	Monocytes	PBL	E^-	E^+
S2C6	1.	<2	<2	<2	10	46	<2
	2.	<2	<2	4	12	44	<2
B1	1.	<2	<2	5	11	47	<2
	2.	<2	3	3	12	43	3
a-Kappa/	1.	<2	<2	NT	11	47	<2
lambda	2.	<2	<2	NT	15	40	3
OKT3	1.	<2	9	7	78	6	90
	2.	<2	31	5	67	5	73
OKM1	1.	<2	79	81	3	25	<2
	2.	<2	69	76	9	30	7

were incubated with a FITC-conjugated rabbit $F(ab')_2$ anti-mouse Ig absorbed with human Ig on Sepharose 4B (Pharmacia). When double labelling was employed to simultaneously detect the sIg^+ population, a TRITC-conjugated rabbit $F(ab')_2$ anti-human Ig was included in this step. These antibodies had been previously absorbed with mouse Ig to exclude reactivity with the monoclonal antibodies. Finally cells were washed and mounted in 50% glycerol, overlayered with cover slips and examined by incident UV light in a Zeiss Universal microscope.

Results

Reactivity with normal haematopoietic cells

To determine the subpopulation of cells in normal blood responsible for binding of the S2C6 antibody, peripheral blood cells were divided into different subpopulations (see Material and methods). As seen from Table 1, showing the results with two representative donors, S2C6 did not stain either erythrocytes, platelets, granulocytes or monocytes. In contrast, a significant portion of highly purified PBL was stained with the antibody. Here, the $S2C6^+$ cells were enriched in the E^- fraction obtained by depletion of cells with receptors for sheep erythrocytes, while the opposite was found for the T cell enriched E^+ fraction. As also seen from Table 1, the S2C6 antigen was in all cases expressed in parallel with both the B1 antigen and surface-bound Ig (as detected by antibodies to Kappa and Lambda). Two-colour double staining for sIg and S2C6 revealed that virtually all sIg^+ cells were also $S2C6^+$. Sometimes a small and varying portion of sIg^- cells was also stained with S2C6 antibody (results not shown). Over a number of tests with eight different donors the portion of $S2C6^+$ cells in PBL ranged from 6% to 15% and in the PBL-E^- fraction from 30% to 65%. The S2C6 antigen was also absent from activated T cells as revealed by testing DR^+ T-blasts from stimulations with Concanavalin A or Leucoagglutinin or from TCGF-dependent long-term T cell cultures.

Cells derived from fresh thymus, spleen and tonsils were also investigated. As seen from Table 2 thymocytes from two different donors were negative for both S2C6 and expression of DR antigens while similar populations of cells obtained from tonsils and from spleen were stained with both S2C6 and DR, respectively.

Table 2. Reactivity of S2C6 antibody with cells isolated from tonsils, spleen and thymus

		Fluorescence (%)	
		$S2C6^+$	DR^+
Tonsil	1.	57	51
	2.	37	32
Spleen	1.	42	48
Thymus	1.	<1	<1
	2.	<1	<1

Reactivity with malignant cells

To investigate the presence of the S2C6 antigen on various malignant lymphoid cells and to resolve the question of a possible stage-specific expression of the antigen during B cell ontogeny, a number of different B cell malignancies was tested. These included non-T ALL, B-CLL, HCL, IC, IC-s and multiple myeloma. As shown in Table 3, all B-CLL, HCL and IC cells were stained with the antibody usually giving a homogenous but fairly weak membrane fluorescence. The number of ALL cases was small but none of the three tested were positive. Two of these were positive for CALLA antigen (as detected by the J5 antibody) and two showed a positive reaction with antibodies to the B1 antigen. Independent experiments with six different cases of non-T ALLs ($CALLA^+$) have confirmed the absence of the S2C6 antigen from this cell type (V. Jønsson, personal communication). Three out of five late stage IC, showing Ig secretion as well as the terminal stage of B cell differentiation, represented by multiple myeloma cells, were not stained with S2C6 antibody. However, two patients with IC-s showed a weak positive staining. In addition to malignant B cells a few cells of T cell origin (T-CLL) and non-lymphoid origin (CML, ANLL) were tested. All of these were negative.

Reactivity with BL lines and EBV-transformed lymphoblastoid cells

In addition to testing freshly isolated malignant lymphoid cells S2C6 was tested against tumour cell lines of haematopoietic origin including a large number of BL lines. With

Table 3. Reactivity of S2C6 with various leukaemic cells

	Fluorescence (%)			
	S2C6$^+$	DR$^+$	OKT3$^+$	Comments
B Cells				
ALL	<2	84	12	Calla$^+$, B1$^-$
ALL	5	54	5	Calla$^+$, B1$^+$
ALL	4	NT	NT	Calla$^-$, B1$^+$
B-CLL	88	NT	<10	B1$^+$
B-CLL	81	NT	<10	B1$^+$
B-CLL	90	93	<10	NT
B-CLL	87	88	<10	B1$^+$
B-CLL	85	94	<10	B1$^+$
B-CLL	86	90	<10	B1$^+$
B-CLL	61	82	16	NT
B-CLL	91	90	<10	B1$^+$
B-CLL	78	94	<10	B1$^+$
B-CLL	82	89	<10	B1$^+$
B-CLL	89	91	<10	B1$^+$
B-CLL	90	89	<10	B1$^+$
B-CLL	62	70	36	B1$^+$
HCL	85	85	14	B1$^+$
HCL	95	97	<10	B1$^+$
HCL	92	88	<10	B1$^+$
HCL	84	86	<10	B1$^+$
IC	68	NT	<10	B1$^+$
IC	78	NT	<10	B1$^+$
IC	50	NT	<10	B1$^+$
IC	92	93	<10	B1$^+$
IC	83	80	<10	B1$^+$
IC	60	NT	<10	B1$^+$
IC-s	90	96	<10	B1$^+$
IC-s	<1	88	<10	B1$^+$
IC-s	6	92	<10	B1$^+$
IC-s	17	NT	NT	B1$^+$
IC-s	75	NT	NT	B1$^+$
Myeloma	<1	12	<10	PC1$^+$, PCA1$^+$
Myeloma	<1	9	<10	PC1$^+$, PCA1$^+$
Non-B cells				
T-CLL	<1	5	97	B1$^-$, OKT4$^-$, 8$^+$
T-CLL	<1	65	86	B1$^-$, OKT4$^-$, 8$^-$
T-CLL	<1	<1	94	B1$^-$, OKT4$^+$, 8$^-$
CML	<1	<1	<10	
CML	<1	9	<10	OKM1$^+$
CML	<1	<1	<10	OKM1$^+$
ANLL	<1	<1	<10	B1$^-$

Table 4. Expression of S2C6 antigen, surface IgM (sIgM) and cytoplasmic IgM (cIgM) on EBV-transformed cells derived from fetal bone marrow and on two cell lines from a pre-B lymphoma

	Positive (%)		
Designation	S2C6	sIgM	cIgM
FeBm-15-E95-STOd1	90	38	25
FeBm-15-E95-STOd2	66	40	17
FeBm-15Mn-E95-STOd1	88	2	40
FeBm-15Mn-E95-STOd3	90	0	11
FeBm-15Mn-E95-STOd7	93	0	0
SPAD-3-STO	100	0	0
SPAD-4-STO	100	0	0

Table 5. Reactivity of S2C6 antibody with heterologous cells

Cells	S2C6$^+$ (%)
Rat spleen	<1
Rabbit spleen	<1
Horse PBL	<1
African green monkey	
PBL	5
PBL-E$^+$	<1
PBL-E$^-$	22
Cynomolgus monkey	
PBL	4
PBL-E$^+$	<1
PBL-E$^-$	19

two exceptions, BL lines gave a strong fluorescence in a high percentage of the cell population (Fig. 1), while neither of four T cell leukaemia lines or the histiocytic line U937 were stained. The two BL lines that failed to give a significant staining (11% for P_3H_3 and 3% for Rael) are known to lack the surface receptor for EBV [9, 28]. However, it has been shown that the P_3H_3 cell line may be induced to express virus receptors [3]. Such induction of the EBV receptor was always accompanied with a simultaneous expression of the S2C6 antigen (>60% of cells S2C6$^+$).

The antibody was also tested against a large number of lymphoblastoid cell lines (LCL) established by EBV-transformation of PBL. These cells, which were either of polyclonal or monoclonal nature consistently gave strong staining of almost all cells. This was also the case with EBV-transformed cells of fetal origin. Thus, as seen from Table 4 five lines obtained by in vitro transformation of fetal bone marrow (Ernberg et al. in prep) were all S2C6$^+$ regardless of their phenotype with respect to Ig expression. Also two established cell cultures (SPAD-3, SPAD-4) of a pre-B phenotype Burkitt-like lymphoma showing Ig gene rearrangement but no cellular or surface Ig (Ernberg et al. in prep) were strongly positive with the antibody.

Reactivity with heterologous cells

The S2C6 antibody was also tested against lymphoid cells of xenogeneic origin. As seen from Table 5 no reactivity was observed with spleen cells from rat or rabbit nor with PBL from horse. However, a small fraction of PBL cells from the African green monkey and Cynomolgus monkeys was stained. Monkey lymphocytes may also be divided into T cell enriched and T cell depleted subpopulations using the same conditions for rosetting with sheep erythrocytes as for human lymphocytes [26]. After such a procedure the S2C6 positive cells were retrieved in the E$^-$ fraction while they were depleted from the E$^+$ cells, strongly suggesting that the monkey cells recognised by the S2C6 antibody were of the B cell lineage.

Discussion

The mouse monoclonal antibody S2C6, derived following immunization with urinary bladder cancer cells, was in-

itially selected for its capacity to discriminate between malignant and normal uroepithelium. This association with bladder tumours has been shown for both cell lines [11] and freshly isolated tissue sections (Ben-Aissa et al. in prep.). In addition to its highly selective expression on bladder tumours the S2C6 antigen was only found on some cells of B lymphocyte origin [11]. In the present study we have confirmed and extended this observation and were able to show that the S2C6 antigen is entirely confined to cells of the B cell compartment within the haematopoietic system. Thus, the antigen was not detected on either granulocytes, monocytes, erythrocytes, platelets or T lymphocytes. In contrast to the association with malignancy observed for urothelial cells the S2C6 antigen appears to be a normal constitutent of B lymphocytes. It was present on peripheral B cells, on B cells from spleen and tonsils and on B cell-derived leukaemias. It was also present on B cells of monkey origin but was absent from horse, rat and rabbit lymphocytes.

By testing different malignant B cells it was possible to relate S2C6 antigen expression to the maturation stage of the B cells. Non-T ALL cells have been described to be pre-B cells as based on expression of the B4 antigen [18] and Ig heavy chain gene rearrangement [12]. None of the nine non-T ALL showed any reactivity with the S2C6 antibody. However, while absent from pre-B cells of the ALL type, S2C6 was expressed in high quantities on cells of progenitor or pre-B cell phenotype obtained by EBV transformation of B lymphocytes from fetal bone marrow. It was present on all cells of intermediate stages, represented by immature cells of B-CLL type, on HCL cells and on the more mature but non-secretory IC cells. However, three out of five IC cells with Ig secretion were negative as well as the terminal stage myeloma cells. This absence of S2C6 from myeloma cells was also observed for the established myeloma line SKO [19]. In contrast, the cell line LicrLon HMy2 [1] derived from a plasma cell leukaemia was strongly reactive. The same distribution on these two cell lines was observed for the B1 antigen. The LicrLon HMy2 has however been shown to be positive for the EBV-induced nuclear antigen, EBNA [1] and it has been suggested to represent a lymphoblastoid cell rather than a true myeloma which would be in line with its phenotypic expression of both B1 and S2C6. Among CLL, HCL and IC, the S2C6 antigen was homogenously expressed on most cells, all being weakly to moderately stained by the antibody. In contrast LCL cells obtained by transformation with EBV always gave rise to a very bright staining suggesting that the antigen expression may be enhanced by EBV infection. However, since these cells were passed in culture the observed increase may also be dependent on the cells being subjected to in vitro conditions. For BL cells all lines except two (Rael and P_3H_3) were strongly positive. The S2C6 antibody stained EBNA negative cells (BJAB, Ramos) as well as EBNA positive lines. A common feature of the two S2C6 negative lines was the lack of receptors for EBV on these cells. Moreover, for one of the two lines (P_3H_3) the cells could be induced to express the virus receptors [3] and this also rendered them S2C6 positive. As the EBV receptor is well characterized and probably identical with the C3d receptor [4] it is, however, clearly distinguishable from the S2C6 antigen. Moreover, pre-treatment of E^- cells with S2C6 antibody was never seen to affect infection and transformation with EBV and, conversely, pre-treatment with EBV did not affect the intensity of staining with the S2C6 antibody (results not shown). However, the possible association of S2C6 with the EBV receptor and its enhanced expression upon EBV infection is of considerable interest and will be further investigated.

Another important matter to be elucidated is the possible relationship of the S2C6 polypeptide to other B cell markers. It appears to be unrelated to MHC class 2 antigens, as tested by antibodies to DR and DC antigens, which are also present on activated T cells and monocytes but absent from bladder tumour cells (results not shown). It is also distinct from the two B cell antigens B1 [25] and B2 [16], the latter being identical with the EBV/C3d receptor [4], as seen from differences in expression on some B cell malignancies as well as from the absence of these antigens on bladder tumour cells. Similar to the S2C6 antigen, Thorley-Lawson et al. [27] have reported on a B cell antigen (B-LAST 1, 45 kdaltons) the expression of which was markedly increased in response to EBV infection. However, in contrast to S2C6, B-LAST 1 was not detected on normal peripheral B cells. By comparison of the molecular weights and specificity patterns reported for other B cell associated antigens [15, 17, 10] it appears likely that the S2C6 antigen constitutes a novel antigen with a very high specificity for B lymphocytes within the haematopoietic system.

The fact that the S2C6 antigen is also present on urothelial carcinomas is intriguing and is being further studied with respect to the possible functional properties of this molecule. With the present knowledge of the products of cellular oncogenes suggesting that some of these may exert their oncogenic potential by acting as growth factors or constitute cellular receptors for such factors (for review see 5) the S2C6 antigen should be investigated for its possible function as the receptor for a B cell growth factor.

Another important task will be to elucidate whether the S2C6 reactive p50 molecule expressed by B cells is identical to that of bladder tumour cells or represents a member of a structurally and functionally related family of molecules. The finding of bladder tumour specific epitopes distinct from that recognised by the S2C6 antibody would be of great importance for the practical use of the p50 molecule as a marker for urothelial tumours. On the other hand, within the haematopoietic system the S2C6 antibody may be useful for the identification of B lymphocytes and for the differential diagnosis of some B cell tumours.

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