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Urinary excretion of CD23 antigen in normal individuals and patients with chronic lymphocytic leukaemia (CLL)

N. R. LING, F. K. STEVENSON* & B. BROWN *Department of Immunology, School of Medical Science, Birmingham and *Tenovus Research Laboratory, Southampton General Hospital, Southampton, UK*

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SUMMARY

A soluble form of CD23 (sCD23) was found in the urine from 12 normal individuals but was not present in 20 normal sera, suggesting that sCD23 produced by cells in tissues is eliminated in the urine. The sCD23 from urine differed in physicochemical properties from the sCD23 found in supernates from B-lymphoblastoid cell lines (B-LCL) and in the sera of patients with B type chronic lymphocytic leukaemia (B-CLL). On SDS–PAGE analysis under reducing conditions urinary sCD23 showed two bands corresponding to molecular weights of 45–60 kD and 28–35 kD indicating that sCD23 may be excreted in combination with another molecule. When subjected to gel filtration in its native state, sCD23 from urine showed a major peak at approximately 150 kD and a minor peak (probably a breakdown product) at 21 kD. Urinary sCD23 was more strongly held by DEAE-cellulose and required 0·5 M buffer pH 8·0 for elution, suggesting that it is more anionic than sCD23 from culture supernates. Five MoAbs recognizing different epitopes on sCD23 from B-LCL supernates were tested on urinary sCD23. Four of the MoAbs were reactive but one (EBVCS-1) was not. Urinary sCD23 did not bind to IgE. The level of sCD23 found in normal urine (approximately 0·02–0·05 μg/ml) was exceeded in 17 of 24 cases of B-CLL. In one case with a high cell count and a serum concentration of 10 μg/ml, the urine contained 80 μg/ml sCD23. In another case a high serum sCD23 was not matched by a high urinary level. In this case the gel filtration pattern was closer to that found with urine sCD23 rather than the B-LCL pattern found with sera of other B-CLL patients.

Keywords CD23 CLL urine

INTRODUCTION

The single chain 45-kD glycoprotein defined at the Second International Workshop on Human Leucocyte Differentiation Antigens as CD23 was first described as an antigen on the surface of cells of B-lymphoblastoid cell lines (B-LCL) by Kintner & Sugden (1981). As well as being strongly expressed on B-LCL, CD23 is also present on the abnormal lymphocytes present in the blood in B-type chronic lymphocytic leukaemia (B-CLL) (Nadler, 1986). CD23 is weakly expressed on resting normal B cells in blood and follicular mantles and is also found on follicular dendritic cells in germinal centres (Ling, MacLennan & Mason, 1987). IL-4 enhances expression of CD23 on B lymphocytes and will induce expression on monocytes (Bonnefoy *et al.*, 1988a; Gordon *et al.*, 1988). At the Third Leucocyte Typing meeting, two groups provided evidence for the identity of CD23 and the low affinity receptor for IgE, FcεRII (Aubry *et al.*, 1987; Kikutani *et al.*, 1987).

Fragments of CD23 are found in culture supernates of B-LCL and arise from proteolytic cleavage of cell surface CD23. Their origin and properties have been reviewed by Gordon *et al.* (1989) and Delespesse, Sarfati & Hofstetter (1989). Soluble fragments of 37, 33 and 25–27 (stable form) kD showing IgE-binding capacity and reactivity with CD23 MoAbs have been identified. Soluble CD23 antigen (sCD23) is present at high levels in B-CLL sera, but at low or undetectable level in normal sera (Sarfati *et al.*, 1988; Lowe *et al.*, 1989; Gordon *et al.*, 1988). The serum level in CLL is related to the tumour load (Lowe *et al.*, 1989). Absence of sCD23 in normal serum should not be interpreted as evidence that normal cells expressing CD23 do not shed fragments which find their way into blood and lymph, but rather that sCD23 is efficiently removed from the blood by the kidney. A form of sCD23 antigen is regularly found in the urine of healthy individuals (Lowe *et al.*, 1989). This study reports an investigation into the properties of urinary sCD23 and the relationship between urinary and serum sCD23 in B-CLL.

Correspondence: Dr N. R. Ling, Department of Immunology, Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

MATERIALS AND METHODS

Sera and urines

Urine samples from normal individuals and patients were treated with sodium azide (final concentration 0·1%) after

collection, stored at 4°C and filtered before testing. Sera from CLL patients and the corresponding urine samples were kindly provided by the East Dorset Pathology Service (Bournemouth, UK). Twenty sera from normal individuals were provided by the Blood Transfusion Centre, Birmingham, UK.

CD23 MoAbs

The mouse MoAb BU-38, isotype G1, was produced in this laboratory and authenticated at the IVth Leucocyte Typing Workshop. The MoAb MHM6, isotype G1, was obtained from Dr M. Rowe (Department of Cancer Studies, University of Birmingham, Birmingham, UK). Three other established CD23 MoAbs, EBVCS-1 (G1), EBVCS-5 (G1) and EBVCS-4 (M) were kind gifts from Dr B. Sugden (McArdle Laboratory, Madison,, WN) and were made available by Dr J. Gordon. Purified IgG fractions of BU-38, MHM6, EBVCS-1 and EBVCS-5 were obtained by chromatography of ascitic fluids on DEAE-cellulose columns.

Affinity purification of CD23 antigens

The CD23 MoAb MHM6 was coupled to activated Sepharose as recommended by the manufacturers (Pharmacia, Uppsala, Sweden). Separate columns were prepared for purification of CD23 from urine or culture supernate. Columns were washed in phosphate-buffered saline containing 0·05% sodium azide (PBS-AZ) and then in 0·5 M NaCl before elution of CD23 with 3 M KCNS. Each 2 ml fraction was titrated for CD23 and positive fractions pooled and dialysed for 2 days against PBS-AZ or gel filtration buffer or 1·2 mM sucrose (samples for freeze-drying and analysis in SDS gels).

Gel filtration and ion-exchange chromatography of CD23 antigens

Gel filtration was performed on 600 × 24 mm diameter columns of S-100 and S-300 Sephacryl (Pharmacia) equilibrated with 0·05 M phosphate buffer pH 7·5 containing 0·2 M NaCl and 0·02% sodium azide. The flow rate was 0·4 ml/min and fractions of 3–4 ml were collected. The columns were calibrated with molecular weight standards supplied by Pharmacia. Samples (0·05 ml) of fractions were titrated for CD23. For ion-exchange chromatography 100 × 12 mm columns of DEAE-cellulose were equilibrated with 0·01 M phosphate buffer pH 8·0 and step elutions with 0·05, 0·10, 0·50 and 1·0 M NaCl containing 0·01 M phosphate pH 8·0 were performed.

^{125}I and ^{35}S labelling procedures

^{125}I. Affinity-purified CD23 from urine or a B-LCL was labelled with ^{125}I by the chloramine-T method as described by Butt (1984). Excess ^{125}I was neutralized by treatment with metabisulphite and potassium iodide. After addition of fetal calf serum (FCS, 0·1%) as carrier, the labelled CD23 was purified by affinity chromatography.

^{35}S-methionine labelling of B-LCL CD23. Cells of LICR-LON-HMy (10^8), an IgA producing B-LCL (Ling *et al.*, 1989) were washed in Hank's balanced salt solution (HBSS) and the cells resuspended to 1×10^6/ml in methionine-free RPMI-1640 medium containing 10% FCS and 500 μCi of ^{35}S-methionine (Amersham International, Amersham, UK). After culture for 2 days the cells were spun down and sCD23 purified from the supernate by affinity and ion-exchange chromotography. Purified, labelled sCD23 preparations were dialysed against 1·2 mM sucrose and freeze-dried for SDS–PAGE analysis.

SDS–PAGE analysis

Freeze-dried samples were taken up in 50 μl of sucrose-free disruption buffer and heated at 100°C for 5 min. Samples were loaded onto 12·5% gels and run for 3–4 h under reducing conditions. Gels were stained, washed, dried and apposed to X-ray film with an intensifying screen for 2 weeks for visualization of radioactive bands.

Titration and epitope analysis of CD23 antigens

Purified MoAbs were coupled to sheep erythrocytes with chromic chloride as already described (Ling, Bishop & Jefferis, 1977; Lowe *et al.*, 1989) and stored at 4°C as a 0·5% suspension in HEPES-buffered RPMI-1640 medium containing the antifungal agent Nystatin (50 U/ml). Since CD23 is a single-chain polypeptide and each epitope has only a single representation, antigen binding but not cross-linking occurs with sheep erythrocytes coated with a single MoAb. Synergistic action of MoAb to spatially distinct epitopes on the antigen is required for cross-linking and agglutination to occur. For routine estimation of sCD23 in fluids and column fractions a 1/1 mixture of sheep erythrocytes coated with MHM6 and sheep erythrocytes coated with EBVCS-5 was used. Unmixed sheep erythrocytes coated with a single MoAb served as a control for non-specific effects. Agglutination and end-points were read after 2–24 h settling. The background in titrations of sera for sCD23 antigen was substantially eliminated by initially diluting the serum 1/5 in 0·1 M 2-mercaptoethanol in saline to dissociate IgM antibodies to sheep erythrocytes and IgM rheumatoid factors. Titrations were standardized against dilutions of a recombinant sCD23 prepared by J.-Y. Bonnefoy (Glaxo, Geneva) from insect cells transformed with baculovirus containing DNA coding for 25K CD23 and kindly provided by Dr J. Gordon. The test was sensitive to 0·5 ng/ml. Epitope analysis was performed by serially diluting each purified CD23 MoAb in a high concentration of affinity-purified CD23 antigen and adding sheep erythrocytes with a complementary MoAb. Prozones and end-points were read after 4 h settling.

IgE binding

A mouse hybridoma secreting a chimaeric IgE MoAb with a human ε constant region heavy chain and mouse anti-NIP variable regions, produced by Neuberger *et al.* (1985) was kindly made available to us by Drs J. Pound and R. Jefferis (Department of Immunology, University of Birmingham, Birmingham, UK). Sheep erythrocytes conjugated with NIP were coated with sub-agglutinating doses of the hybrid MoAb. Strong rosettes were formed when IgE-coated sheep erythrocytes were lightly centrifuged with a CD23-expressing B-LCL (20/1 ratio) and resuspended by gentle inversion of the tube. A rosette-inhibition test similar to that described by Barsoumian *et al.* (1989) and Sarfati *et al.* (1987) was used to test for IgE-binding properties of urine sCD23 preparations. For this test a 2·5% suspension of IgE-coated sheep erythrocytes (50 μl) and B-LCL cells (50 μl, 2×10^5 cells) was added to a small plastic tube containing SCD23 antigen affinity-purified from urine (0·1–1·5 μg in 0·25 ml of HEPES-buffered RPMI medium containing 2% FCS) and centrifuged at 280 *g* for 3 min. Rosettes were counted after gentle resuspension.

Another test system used was similar to that successfully employed to demonstrate binding of sCD21 to C3d and Epstein–Barr virus (EBV) (Ling *et al.*, 1991). It tested for a

bridging effect of affinity-purified IgE on sheep erythrocytes coated with a single CD23 MoAb and then exposed to excess CD23 antigen.

RESULTS

sCD23 in the urine of CLL patients and normal individuals

sCD23 was present in all 12 urine specimens from normal individuals, but at a low level (μg/ml urine = 0·02, 0·03, 0·03, 0·004, 0·03, 0·05, 0·03, 0·02, 0·03, 0·02, 0·01, 0·02). Similar low levels were recorded from two of 12 samples from CLL patients but in the other 10 cases CD23 was present at moderate, high or very high levels (μg/ml urine in the 12 specimens = 0·008, 2·0, 0·13, 1·0, 0·5, 0·5, 50·0, 0·13, 0·25, 4·0, 0·26, 0·03). Serum samples were also examined. sCD23 was not detectable (< 0·01 μg/ml) in 20 normal sera but was present in sera of CLL patients as previously reported (Lowe *et al.*, 1989). On the basis of these preliminary results a study of serum and urine sCD23 in 24 cases of CLL phenotyped for cell surface CD23 was planned.

Comparison of urine and serum levels of sCD23 in CLL

In 17 out of 24 sera from unselected cases of CLL the serum sCD23 was increased (⩾0·01 μg/ml) and in all but one of the 17 the urine sCD23 was also increased (Fig. 1). In five of the seven cases without raised serum sCD23, urine sCD23 was raised (> 0·12 μg/ml). In one case (patient 1) outstandingly high levels of serum and urine sCD23 accompanied the presence of a large number of CD23-expressing tumour cells in the blood. In another case (patient 2) with a high level of serum sCD23, a correspondingly high urine sCD23 was not observed. The sCD23 in this case was further examined by gel filtration (see later).

Molecular weight determinations of sCD23 in SDS gels

The sCD23 produced by cells of the B-LCL LICR-LON-HMy grown in the presence of ^{35}S-methionine showed bands in the 30-kD and 25–27-kD region in agreement with published reports (Sarfati *et al.*, 1987; Delespesse *et al.*, 1989). Bands in the 25–27-kD region were also obtained from ^{125}I-labelled affinity-purified sCD23 supernate (band 8 in Fig. 2) whereas ^{125}I-labelled affinity-purified sCD23 from normal urine showed bands in the 45–60-kD and 28–35-kD regions (bands 9 and 10 in Fig. 2). One possibility considered is that band 10 is sCD23 and band 9 is a protein which remained associated with it during affinity purification. sCD23 affinity-purified from a pool of CLL sera also showed bands in the 25–27-kD region, but only a faint band in the 45–60-kD region (not shown).

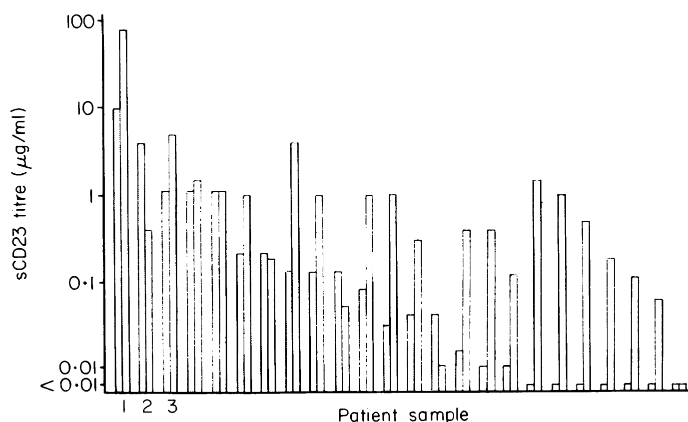


Fig. 1. Comparison of serum and urine levels of sCD23 in 24 cases of B-CLL. In each pair the serum titre is on the left and the urine titre on the right. The leucocyte count of patient 1 was 149 × 10^6/ml and 46% of the cells were positive for CD23 by flow cytometry. The corresponding values for patient 2 were 27 × 10^6/ml and 83% and for patient 3 34 × 10^6/ml and 23%.

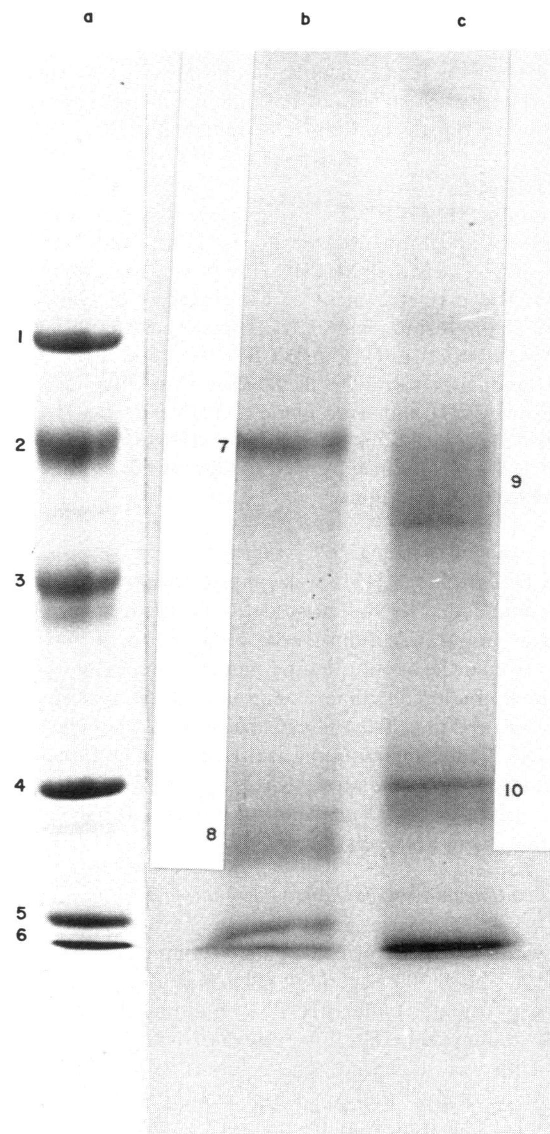
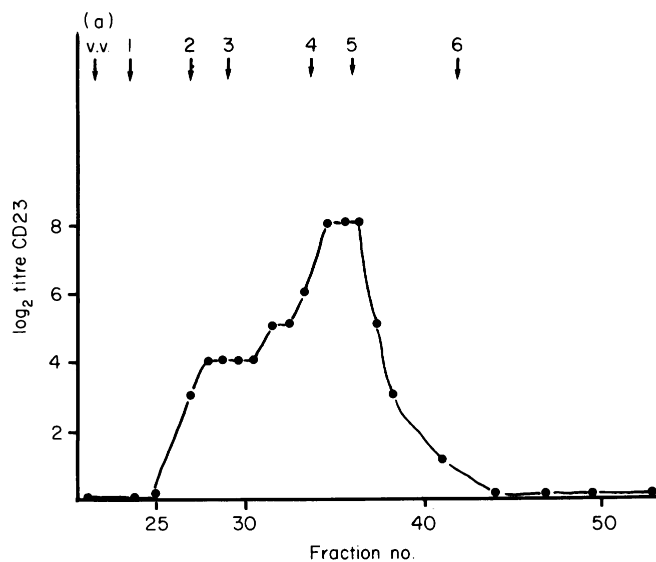


Fig. 2. SDS gel analysis of ^{125}I-labelled sCD23 affinity-purified from B-LCL supernate (track b) and from urine (track c); 12·5% gel run for 3·5 h with 2-mercaptoethanol (2ME) present. The disruption buffer contained 5% 2ME. Molecular weight standards, stained with Coomassie Blue (track a): 1 = phosphorylase B (94 000); 2 = bovine albumin (67 000); 3 = ovalbumin (43 000); 4 = carbonic anhydrase (30 000); 5 = trypsin inhibitor (20 100); 6 = α-lactalbumin (14 400). The autoradiographs shown in tracks b and c were developed for 2 weeks. In track b, 7 = albumin contaminant, 8 = B-LCL sCD23 bands. In track c, 9 and 10 = urinary sCD23 bands.

Gel filtration analysis of native forms of sCD23 from B-LCL, urine and B-CLL sera

Further differences between urine and B-LCL derived sCD23 became apparent when material from the two sources was



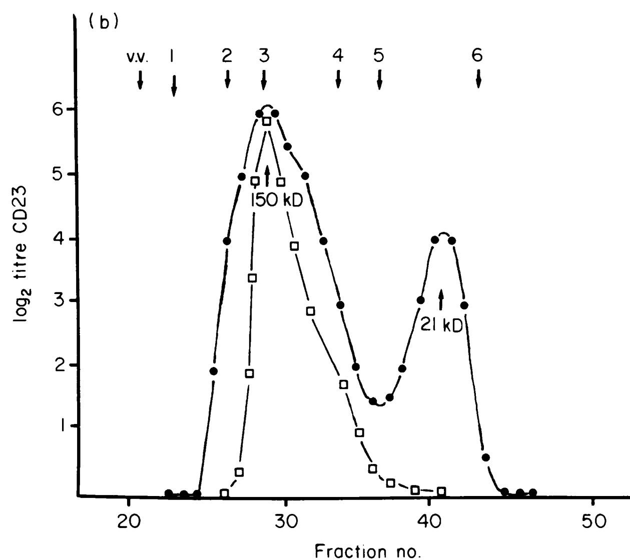
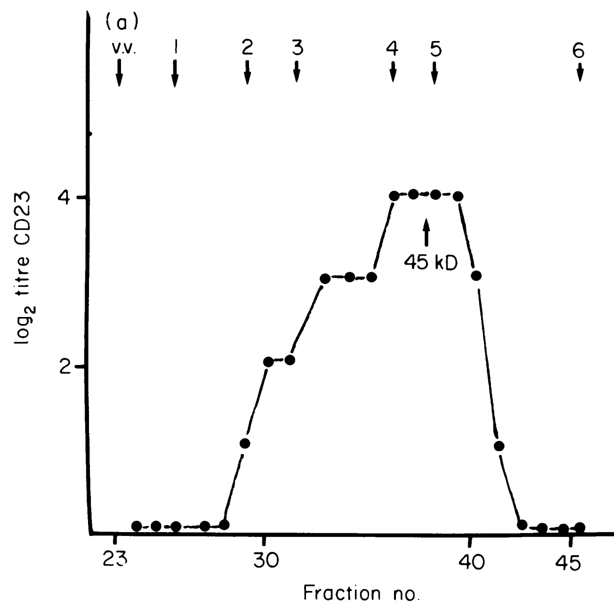


Fig. 3. Gel filtration analysis of sCD23 affinity-purified from a B-LCL (a) or normal urine (b). □, sCD23 isolated from fresh urine, ●, from stored urine. v.v., void volume; 1, ferritin (440 000); 2, catalase (232 000); 3, aldolase (158 000); 4, bovine albumin (67 000); 5, ovalbumin (43 000); 6, ribonuclease (13 700); bed volume of Sephacryl S-300 column = 262 ml.



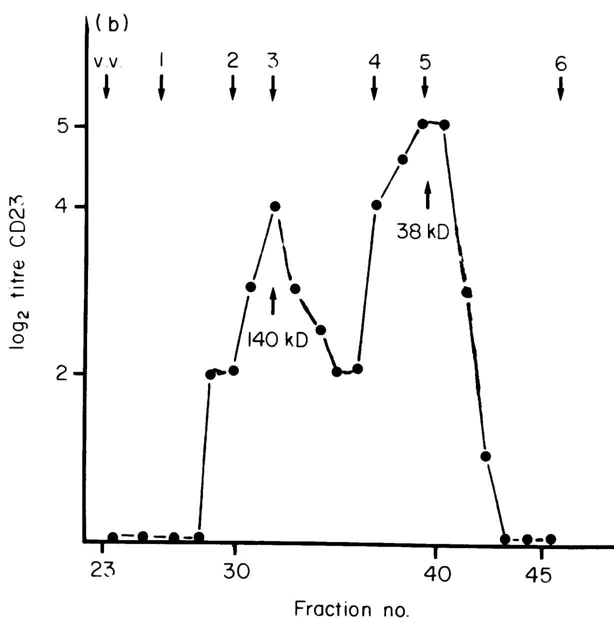


Fig. 4. Gel filtration analysis of sera from two CLL patients, patient 3 (a) and patient 2 (b). A mixture of serum (0·5 ml) and saline (0·5 ml) was dialysed against running buffer before introduction into the column. Other details as for Fig. 3.

examined in its native state by gel filtration. Purified B-LCL sCD23 showed a broad spread from approximately 20 kD to 232 kD with a peak in the 40–60-kD region (Fig. 3a). Fresh untreated B-LCL supernate gave a similar pattern. In contrast, the sCD23 from normal urine showed two clear peaks—a major one at approximately 150 kD and a minor peak at approximately 21 kD (Fig. 3b). A pool of CLL urines gave a similar plot. The 21-kD material was absent or present in only small amounts in sCD23 isolated from fresh urine, but increased on storage of the urine, indicating that it is probably a break-down product. Negative results with a single MoAb indicated that aggregation would not account for the high molecular weight of the principal component. Association with another protein was considered more likely.

Two CLL sera selected for analysis showed distinctly different patterns. In one case (patient 3, Fig. 4a) a broad spread of sCD23 with a mean at approximately 45 kD was observed. This pattern had previously been obtained with a pool of CLL sera and is probably the typical pattern. It resembles the result obtained with supernates of B-LCL cultures (Fig. 3a). The chromatogram obtained in the other case (patient 2, Fig. 4b) showed peaks at approximately 38 kD and 140 kD and is closer to the urinary than the B-LCL pattern. It is of interest that this

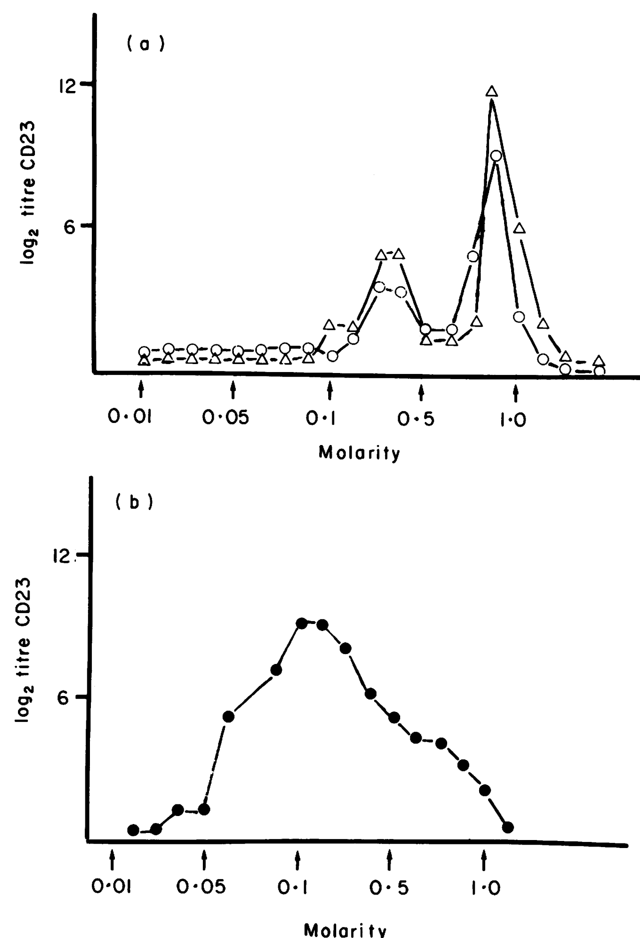


Fig. 5. Chromatography on a DEAE-cellulose column of affinity-purified sCD23 from two samples of urine, ○ and △ (a) and a B-LCL supernate (b).

patient showed an unusually high level of serum sCD23 without a comparable increase in urinary sCD23.

The apparent higher molecular weight of the urinary form of sCD23 compared with that in cell supernates could be due to association with a large molecule during passage through the kidney. In view of the known lectin-like properties of CD23, the associating molecule could be a glycoprotein or polysaccharide. Immunohistological studies of normal renal tissue did not reveal any sites of capture or synthesis of sCD23. The only staining seen was over the glomerulus; this was faint and could have arisen from serum material being filtered through.

Ion-exchange chromatography of sCD23 from a B-LCL and urine
The sCD23 from a B-LCL was eluted from DEAE-cellulose with a single peak at 0·1 M phosphate buffer pH 8·0 (Fig. 5b). Urinary sCD23 eluted with a minor peak at 0·1 M and a major peak at 0·5 M buffer (Fig. 5a). This was interpreted as an indication that the urinary sCD23 is more anionic than B-LCL sCD23 resulting from acylation of amino groups or introduction of negative groups.

Epitope analysis of sCD23 from urine and a B-LCL
Urinary and B-LCL sources of CD23 antigen were compared for their capacity to bind MoAb to different epitopes. A complementary CD23 MoAb was used as an indicator of binding. The results are shown in Table 1. The epitopes detected by MoAb EBVCS-4, EBVCS-5, MHM6 and BU-38 appeared to be available on sCD23 from B-LCL cell supernates and urine, whereas the epitope detected by MoAb EBVCS-1 was available on sCD23 from the B-LCL but not that from urine. A similar result has been obtained in an ELISA-based assay (J. Gordon, personal communication).

Failure to demonstrate IgE-binding properties of urinary sCD23
Sheep erythrocytes derivatized with NIP and coated with

Table 1. Titration of pairs of CD23 MoAbs against sCD23 antigen from urine and a B-LCL

Serially diluted CD23 antibody	Source of CD23 antigen in diluent (0·5 μg/ml)	Indicator CD23 antibody on sheep erythrocyte	Agglutination pattern and corresponding antibody concentration (μg/ml)		
			Prozone down to	Range of maximum agglutination	End point
EBVCS-1	LCL	MHM6	30	1·0–0·25	0·06
	Urine	MHM6	None	nil	nil
	LCL	BU-38	8·0	4·0–0·06	0·03
	Urine	BU-38	None	nil	nil
EBVCS-5	LCL	MHM6	None	10·0–0·04	0·01
	Urine	MHM6	None	10·0–0·04	0·01
	LCL	BU-38	None	10·0–0·04	0·02
	Urine	BU-38	None	10·0–0·04	0·02
MHM6	LCL	EBVCS-5	15	2·0–0·04	0·02
	Urine	EBVCS-5	4	1·0–0·02	0·01
EBVCS-4	LCL	EBVCS-5	None	10·0–0·04	0·01
	Urine	EBVCS-5	None	10·0–0·04	0·01

Free MoAb (DEAE purified) was serially diluted in 0·05 ml volumes of HEPES-buffered RPMI medium containing 2% FCS and 0·5 μg of sCD23 from LCL or urine as shown in column 2. The indicator MoAb coupled to sheep erythrocytes was directed against a different determinant. Agglutination patterns were read after 4 h. Note the negative results of urine CD23 with MoAb EBVCS-1.

chimaeric IgE MoAb showed strong rosettes when tested against B-LCL cells expressing CD23 antigen. No rosettes were obtained with control uncoated NIP-sheep erythrocytes. Inhibition of rosette formation was not observed even with a high concentration (5 μg/ml) of affinity-purified urinary sCD23. Reducing the strength of the rosettes by using lower coating doses of IgE MoAb did not alter the result. Rosettes were observed with coating doses of IgE MoAb in the range 1/2 to 1/32 dilution but no evidence of inhibition was obtained with urinary sCD23 present. In a different test system sheep erythrocytes were coated with a single sCD23 MoAb and saturated with urinary sCD23 antigen. Agglutination tests for a bridging effect of IgE were then performed and were uniformly negative whichever CD23 MoAb was used for priming the sheep erythrocytes.

The possibility that urinary CD23 was excreted in a complex with IgE was considered. Urine was passed through an affinity column of Sepharose-bound polyclonal sheep anti-human IgE. The sCD23 content in the effluent was not diminished and no sCD23 could be recovered from the column. Also, preparations of affinity-purified urinary CD23 did not contain IgE.

DISCUSSION

The tissue source of urine sCD23 has not been identified, but it is likely to be lymphoid tissue which contains two cell types, B lymphocytes and follicular dendritic cells, expressing CD23. Other surface receptors which have been found in soluble form in urine include IL-2R (Marcon *et al.*, 1988), IL-6R and IFN-γ-R (Novick *et al.*, 1989) and a protein-binding tumour necrosis factor (Engelman *et al.*, 1989). The IL-2R was shown to be of molecular weight 40–45 kD and the IFN-γ-R 40 kD. The sCD23 released by cultured cells *in vitro*, and probably by CD23-expressing cells *in vivo* is also of low molecular weight (25–30 kD) and would be expected to be filtered out in the kidney. Thus the presence of sCD23 in urine of normal individuals and its absence from serum is not surprising. However, the urinary form appears to be of larger size and have different properties to the material released by cells and thus appears to have undergone modification before excretion.

There is increasing realization that cell surface receptors and fragments shed from cells frequently exist in non-covalent association with other molecules and that this may have functional significance. It has been shown that CD23 and HLA-DR antigens are spatially associated on the cell surface (Bonnefoy *et al.*, 1988b). Our gel filtration results reveal that the forms of sCD23 found in serum and culture supernates cover a size range of 20–232 kD with a peak at approximately 40–60 kD compared with 25–30 kD by SDS-PAGE analysis. This could be due to the fact that protein–protein complexes already existing on the cell surface are retained or that new complexes are formed after shedding (e.g. with serum proteins). These complexes would be disrupted by SDS denaturation. Excreted sCD23 appears to become associated with another molecule during passage through the kidney. The associated molecule is unlikely to be HLA-DR which would show two extra bands of 36 kD (α chain) and 28 kD (β chain) on SDS analysis, whereas the associated molecule found appears to be of 45–60 kD and its nature is unknown. It associates in such a way that at least one epitope is covered and IgE-binding capacity is lost.

In the IVth Leucocyte Typing Workshop, CD23 MoAbs were classified into three groups based on epitope analysis (Schwarz-Albiez & Moldenhauer, 1989). Two of the MoAbs used in this study (MHM6 and BU-38) fell into one of the groups but the EBVCS MoAbs were not tested. Our preliminary results would indicate that epitope testing of the large number of CD23 MoAbs now available (including the EBVCS MoAb) against the various forms of CD23 antigen would be profitable.

The sCD23 found in the serum of patients with B-CLL is very similar in properties to that released by cultured B-LCL cells and would also be expected to be excreted in urine. The raised levels of urinary sCD23 found in B-CLL confirm that this occurs. In one case, however, a high serum sCD23 was recorded with relatively little sCD23 in urine. Failure of excretion could not be accounted for by renal dysfunction. When examined in its native state by gel filtration the serum size-profile of sCD23 of this patient was closer to the urinary than the serum/cell supernate pattern, perhaps indicating an accumulation of the urinary form in the circulation.

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