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Evidence for a change in the expression of β_2-microglobulin-associated membrane structures on leukaemic human cells

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SUMMARY

Cell-associated and serum β_2-microglobulin was estimated in seven patients with chronic lymphocytic leukaemia. The amount of cell-associated β_2-microglobulin was significantly reduced ($P<0{\cdot}01$), due to a decrease in the fraction of β_2-microglobulin that passes unretarded through a concanavalin A affinity column (presumably non-HLA-associated β_2-microglobulin).

Serum concentrations of β_2-microglobulin were increased, but no correlation was found between the decrease in cell-associated β_2-microglobulin and the increase in serum β_2-microglobulin. All of the β_2-microglobulin from leukaemic serum was eluted corresponding to a molecular weight of 11,800 and none of it was retarded on a concanavalin A affinity column.

The decrease in cell-associated β_2-microglobulin might reflect a change in the qualitative or quantitative expression of β_2-microglobulin-associated membrane structures on the leukaemic cells, perhaps conferring resistance to the cells against hypothetical immunological host defence mechanisms.

INTRODUCTION

β_2-microglobulin is a low molecular weight (11,800) human protein (Berggård & Bearn, 1968) found on cell membranes in association with HLA antigens as a non-covalently bound light chain on the heavier (mol. wt. 45,000) alloantigenic molecule (Cresswell *et al.*, 1974; Grey *et al.*, 1973; Nakamuro, Tanigaki & Pressman, 1973; Neauport-Sautes *et al.*, 1974; Oestberg, Lindblom & Peterson, 1974; Peterson, Rask & Lindblom, 1974; Poulik, Bernoco & Ceppellini, 1973; Solheim & Thorsby, 1974; Tanigaki *et al.*, 1973). Various studies seem to indicate that β_2-microglobulin on human cell membranes is found in excess of HLA antigens (Dorval *et al.*, 1977; Neauport-Sautes *et al.*, 1974; Plesner, 1976; Solheim & Thorsby, 1974), but nothing is known about the structure and function of other cell membrane constituents associated with β_2-microglobulin. However, the immunoglobulin-like structure of β_2-microglobulin points towards an important function for this and associated protein molecules in cell-to-cell interactions (Smithies & Poulik, 1972).

A counterpart to β_2-microglobulin is found in various animals (Apella *et al.*, 1976; Berggard, 1974; Cunningham & Berggard, 1975; Henning *et al.*, 1976; Natori *et al.*, 1974, 1975; Peterson *et al.*, 1975; Schwartz *et al.*, 1976; Silver & Hood, 1974; Smithies & Poulik, 1972). In mice, β_2-microglobulin has been shown to be associated exclusively with H2 and Tla antigens (Geib *et al.*, 1976; Vitetta *et al.*, 1976).

In this study, β_2-microglobulin is used as an indicator of the presence of membrane structures of presumed immunological significance on lymphocytes from patients with chronic lymphocytic leukaemia

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(CLL). The purpose was to learn if the expression of these molecules on leukaemic cells is changed, leaving the cells less susceptible to immunological host defence mechanisms.

Increased concentrations of β_2-microglobulin are found in the serum from patients with advanced malignancies (Evrin & Wibell, 1973). The possible relationships between the amount of cell-associated β_2-microglobulin and serum concentrations, and some biochemical properties of β_2-microglobulin in the serum from leukaemic patients, are included in this study.

MATERIALS AND METHODS

Patients. β_2-microglobulin was measured in the lymphocytes and serum from seven patients (age: 63–79 years) with CLL. The diagnoses were established on the basis of clinical data, blood and bone marrow examination, and the duration of disease before the study was 1 month to 6 years.

The number of lymphocytes in the blood varied from 2 to 58×10^9/l.

In four patients no treatment was given at the time of investigation, whereas three patients (H.A.C., A.H., J.P.P.) were on treatment with prednisone (doses: 7·5–30 mg per day) and one patient (A.H.) received irradiation of the spleen. Treatment with chlorambucil had been discontinued immediately before the investigation in one patient (A.H.), and two other patients (H.A.C., G.B.) had previously been treated with chlorambucil. All patients had normal serum creatinine values.

Isolation of lymphocytes. Heparinized venous blood was collected under sterile conditions and diluted 1:1 with RPMI 1640 culture medium (Gibco Bio-Cult, Great Britain). Lymphocytes were isolated by gradient centrifugation on Lymphoprep® (Nyegaard and Co., Oslo, Norway), washed repeatedly and quantified in a haemocytometer chamber. To minimize the possibility that differences in the expression of cell-associated β_2-microglobulin were due to regeneration of cell membrane constituents after separation of cells from plasma, control experiments were performed in the following way: lymphocytes were isolated in parallel at 4°C and room temperature, or were isolated at 4°C, and incubated in RPMI 1640 for 3 hr at 4°C and at room temperature at a cell density of 10^6 cells per ml culture medium.

Preparation of enriched B- and T-lymphocyte populations. T and B lymphocytes were separated by means of rosette sedimentation (Jondal, 1974). In short, lymphocytes were incubated with washed sheep erythrocytes (SRBC) in heat-inactivated (56°C for 1 hr) foetal calf serum (FCS). The ratio between lymphoid cells and SRBC was 1:10. The mixture was centrifuged at 200 *g* for 6 min, then incubated first for 15 min at 37°C and then for 45 min at 4°C. The cell pellet was then carefully suspended and centrifuged (600 *g*) on a Ficoll–Isopaque gradient. The T cells forming spontaneous rosettes with SRBC (E-RFC) sedimented, whereas other cells (including B cells) stayed in the fluid interface. The former cells were called T lymphocytes after lysis of SRBC with 0·84% ammonium chloride and the latter cells were called B lymphocytes.

T lymphocytes contained more than 80% E-RFC and less than 1% EA- or EAC-RFC, whereas B lymphocytes contained more than 60% EA- or EAC-RFC and less than 5% E-RFC (Jensen, Kurpiaz & Rubin, 1977).

Serological and functional characteristics of leukaemic lymphocytes. The methods for enumeration of B and T lymphocytes have been described in detail elsewhere (Platz *et al.*, 1976). In short, the isolated leukaemic lymphocytes were either incubated with carbonyl-iron powder to remove phagocytosing cells (for the rosette techniques) or incubated with latex particles to make the phagocytosing cells visible in the microscope (for the fluorescence technique).

As the T-cell markers spontaneous rosette formation with sheep erythrocytes (E rosettes) was used and as the B-cell markers rosette formation with human A erythrocytes incubated with rabbit anti-A antibody and mouse complement (EAC rosettes) or membrane fluorescence with FITC-conjugated rabbit anti-human Ig of the following specificities: polyvalent, anti-kappa and anti-lambda, and anti-IgG, -IgM and -IgA were employed. HLA-A, -B and -C typing was performed by a microlymphocytotoxicity method (Kissmeyer-Nielsen & Kjerbye, 1967).

Lymphocyte transformation studies *in vitro* were done with a number of mitogens and microbial antigens in different concentrations, as described earlier (Platz *et al.*, 1976).

Solubilization of cell-associated proteins and affinity chromatography on concanavalin A–Sepharose. These techniques have been described in detail previously (Plesner, 1976). Briefly, isolated lymphocytes were solubilized in non-ionic detergent (Berol, MoDo Kemi, Stenungsund, Sweden) at a maximal cell density of 6×10^7 cells per ml. Low molecular weight carbohydrate is separated from protein by gel filtration on Sephadex G-25 fine (Pharmacia, Uppsala, Sweden), followed by separation of proteins by affinity chromatography on concanavalin A–Sepharose (Pharmacia) (Con A–Sepharose).

Radioimmunoassay of β_2-microglobulin. The amount of β_2-microglobulin in cell extracts and Con A-reactive and Con A non-reactive fractions is estimated by a competitive radioimmunoassay (Plesner, Nörgaard-Pedersen & Boenisch, 1975). Con A-reactive β_2-microglobulin is assumed to represent HLA-associated β_2-microglobulin (called Con A β_2-microglobulin) (Snary *et al.*, 1974, 1976), whereas Con A non-reactive β_2-microglobulin (called non-Con A β_2-microglobulin), might be associated with other protein molecules of unknown structure and function.

Examination of serum from leukaemic patients. Serum was collected and kept at −20°C until further analysed. β_2-microglobulin in serum was estimated by radioimmunoassay (Plesner *et al.*, 1975) after dilution (1:500 in phosphate buffer: 0·1 mol/l sodium phosphate, 0·05 mol/l sodium chloride, 0·006 mol/l EDTA, 0·005 mol/l sodium azide, 1·0 g/l bovine serum albumin (Sigma, St Louis, U.S.A.), pH 7·4. Two sera with high concentration of β_2-microglobulin were further analysed by gel filtration on Sephadex G-75 (Pharmacia) and by affinity chromatography on Con A–Sepharose. In the gel filtration experi-

ments 3 ml serum samples were separated on column K 26/100 (Pharmacia) packed with Sephadex G-75, equilibrated with the phosphate buffer described above. The effluent was collected in 200 2·5 ml fractions and the β_2-microglobulin concentration in each fraction was determined by radioimmunoassay. The elution volume of β_2-microglobulin was compared to the elution volume of cytochrome C (mol. wt. 12,400) (Boehringer–Mannheim, W. Germany). Affinity chromatography was performed as described for cell extracts (Plesner, 1976), except that detergent was not included in the buffer. Protein corresponding to 50 μl of serum was separated on 5 ml columns of Con A–Sepharose.

Healthy controls. A total of thirteen estimates of cell-associated β_2-microglobulin, including the estimation of non-Con A β_2-microglobulin and Con A β_2-microglobulin, was made on seven healthy individuals aged from 27 to 65 years. No difference due to age was found. The reference population with respect to serum β_2-microglobulin has been characterized previously (Plesner *et al.*, 1975). The standard deviation was calculated according to this formula:

$$\text{s.d.} = \left(\frac{\sum_{i=1}^{i=N} (x_i - \bar{x})^2}{N-1} \right)^{\frac{1}{2}}.$$

Statistical evaluation of results. The amount of cell-associated β_2-microglobulin in healthy individuals and leukaemic patients was compared by the Mann–Whitney rank sum test. The correlation between serum β_2-microglobulin and cell-associated β_2-microglobulin in leukaemic patients was examined using the least squares method.

RESULTS

The classification of leukaemic lymphocytes according to cell surface markers and the corresponding lymphocyte concentration is shown in Table 1. It appears that all the patients have a low relative

TABLE 1.

Patient	Date of examination (1976–77)	Rosettes		Immunofluorescence						Blood lymphocytes ($\times 10^{-9}$/l)
		E	EAC	Polyvalent	κ	λ	G	A	M	
E.N.	20/7	23	21	75						40
	3/8	13	19	90						51
H.R.	31/8	10	37	90		90			90	58
J.P.P.	17/8	11	25	100						24
	19/10	20	25	90	90				90	31
J.L.	22/6	9	35	90						15
	14/9	15	35	90						13
H.A.C.	11/11	5	15	90	90					12
A.H.	2/12	25	53	80	80		5		80	2

concentration of T cells (normally above 52%), although the absolute concentration is not decreased. The concentration of B cells is highly elevated, and this is especially the case when the Ig-positive cells are taken into account.

By the lymphocyte transformation test we found a decreased incorporation of labelled thymidine compared to healthy individuals (results not shown).

The amount and distribution of cell-associated β_2-microglobulin and the concentration of β_2-microglobulin and the concentration of β_2-microglobulin in the serum from leukaemic patients is shown in Table 2. Total and non-Con A β_2-microglobulin is significantly reduced on leukaemic lymphocytes ($P<0{\cdot}01$), whereas Con A β_2-microglobulin is unchanged ($P>0{\cdot}10$). No difference is found between cells isolated or incubated at 4°C or at room temperature. The decrease is not due to the B-cell nature of the CLL lymphocytes, since B lymphocytes from a healthy individual express the same amount of β_2-microglobulin as unseparated lymphocytes.

TABLE 2.

Patient	Date of examination (1976–77)	Cell-associated β_2-microglobulin molecules per lymphocyte ($\times 10^{-5}$)			β_2-microglobulin in serum (nmol/l)
		Total	Non-Con A	Con A	
E.N.	20/7	2·8	1·6	1·2	220
	3/8	2·3	1·6	0·7	235
	16/11	5·2	4·1	1·1	370
	4/1	1·7	1·0	0·7	358
	4/1*	1·5	0·9	0·6	—
	4/1*	1·6	1·0	0·6	—
H.R.	1/6	5·6	3·4	2·2	200
	31/8	2·2	0·8	1·4	165
	30/11	2·6	1·7	0·9	360
J.P.P.	17/8	5·1	3·8	1·3	310
	19/10	4·0	2·4	1·6	235
	2/11	4·4	3·4	1·0	255
J.L.	22/6	3·6	2·2	1·4	205
	14/9	2·8	1·7	1·1	255
H.A.C.	11/11	3·2	2·2	1·0	468
	9/12	4·0	2·4	1·6	447
A.H.	4/11	3·2	2·6	0·6	583
	2/12	4·6	3·2	1·4	475
G.B.	16/12	2·4	1·4	1·0	580
	16/12†	2·2	1·3	0·9	—
Healthy individuals (mean ± 2 s.d.)		5·2 ± 1·7	3·9 ± 1·5	1·3 ± 0·6	147 ± 52
B lymphocytes (mean of two)		5·2	4·1	1·2	
T lymphocytes (mean of two)		3·8	2·8	1·0	

* Incubated at 4°C and 20°C.
† Isolated at 4°C.

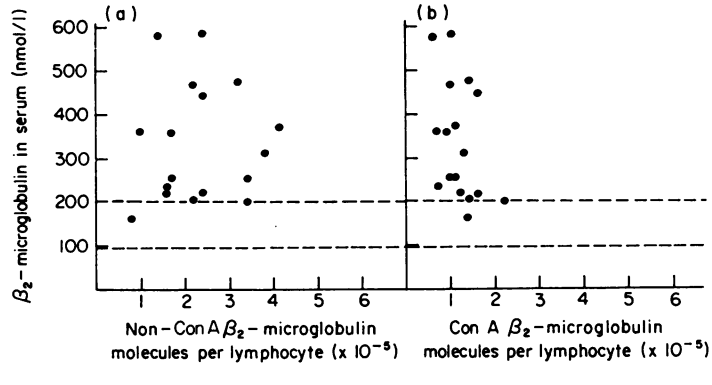

FIG. 1. Correlation between lymphocyte-associated and serum β_2-microglobulin in patients with chronic lymphocytic leukaemia. (a) Non-Con A β_2-microglobulin; $r = 0{\cdot}085$. Dashed lines show normal mean ± 2 s.d. (b) Con A β_2-microglobulin; $r = -0{\cdot}40$. Dashed lines show normal mean ± 2 s.d.

As shown in Fig. 1 no correlation is found between the concentration of β_2-microglobulin in the serum from leukaemic patients and the amount of non-Con A β_2-microglobulin or Con A β_2-microglobulin on leukaemic lymphocytes.

β_2-microglobulin from the serum of leukaemic patients was eluted in a single peak after gel filtration, corresponding to a molecular weight of 11,800. By affinity chromatography all of the β_2-microglobulin was recovered in the Con A non-reactive fractions.

DISCUSSION

The finding of an expansion of the B-cell subset in CLL is in accordance with the findings of other authors (Aisenberg & Bloch, 1972; Kubo, Grey & Pirofsky, 1974; Nies *et al.*, 1973; Preud'homme & Seligman, 1972; Rudders, 1976; Wibran, Chandler & Fudenberg, 1973) and so is the monoclonal distribution of these B cells. The discrepancy between the number of B cells estimated by the EAC rosette technique and membrane fluorescence is a common finding in our laboratory when investigating patients with CLL having a high concentration of peripheral blood lymphocytes (Thomsen *et al.*, in preparation). This is in contrast to the findings in healthy individuals and patients with non-haematological disorders.

One major pitfall in the estimation of the number of Ig-positive cells by a fluorescence technique is the unspecific binding of immunoglobulins to the Fc receptor of cells, not necessarily B cells. Firstly, the immunoglobulin of the patients' serum may be passively absorbed onto Fc receptors of the cells, and secondly, the Fc receptor might bind the fluorescein-conjugated antiserum itself. In both cases, the monoclonal distribution of the immunoglobulins were 'blurred' by non-specific binding of the immunoglobulins, but this was not found to be a major problem, possibly due to a careful washing of the cells to be tested in the serum-free medium (to remove passively absorbed immunoglobulins) and the filtration of the fluoresceinated antiserum immediately before use in order to avoid aggregates. Thus we did not find it necessary to use $F(ab')_2$ fragments of rabbit antisera or to take further precautions to get rid of non-specific bound Ig.

The results show variations from patient to patient in the distribution of markers. Sequential studies have been made by others (Davis, 1976), demonstrating that the pattern of circulating subpopulations may vary greatly with time.

It has not been possible so far to determine any variations in the expression of HLA antigens on the surface of the lymphocyte. The microlymphocytotoxicity test employed is probably not optimal for such studies, but by careful titrations of HLA antisera we hope to be able to define possible fluctuations in the individual patient.

The finding of a reduced amount of cell-associated β_2-microglobulin on leukaemic cells is of interest, since it most likely reflects a change in the expression of membrane structures of immunological significance on the leukaemic cell membrane, perhaps conferring some degree of resistance on the cells against hypothetical immunological host defence mechanisms. The reduction is variable from time to time in the same individual and further studies will be needed to learn if this has any clinical significance. The variations in the amount of cell-associated β_2-microglobulin does not seem to be an *in vitro* phenomenon (e.g. *in vivo* antigenic modulation with variable degrees of regeneration of cell surface components *in vitro*), since incubation in parallel at 4°C and at 20°C does not result in any difference in the amount of cell-associated β_2-microglobulin.

It will be of interest to learn if the decrease in cell-associated β_2-microglobulin is a general finding in other types of leukaemia, and whether immature lymphocytes, such as mitogen-induced blast cells and the cells found in infectious mononucleosis, differ from leukaemic cells. We have, indeed, preliminary data indicating that the leukaemic cells in acute lymphocytic and acute myelocytic leukaemia do express smaller amounts of non-Con A β_2-microglobulin and that Con A-induced lymphoblasts and 'lymphoblasts' from patients with infectious mononucleosis express increased amounts of β_2-microglobulin.

The separation of cell-associated β_2-microglobulin in non-Con A and Con A fractions was provoked by the finding that some human HLA antigens (first and second series) are glycoproteins capable of combining with Con A (Snary *et al.*, 1974, 1976). Recent data obtained in experiments with certain

mouse strains indicate that all β_2-microglobulin on blood lymphocytes is associated with H2 antigens (Geib *et al.*, 1976). However, estimations of β_2-microglobulin and HLA antigens on human cells point towards a 2–3 molar excess of β_2-microglobulin over HLA antigens (Dorral *et al.*, 1977; Plesner, 1976). Perhaps the present 'one light chain: one heavy chain' model for human alloantigens will have to be re-evaluated. If it turns up to be invalid and is replaced by a three or four chain model, one HLA heavy chain being associated with two or three β_2-microglobulin light chains, our data seem to indicate that the β_2-microglobulin molecules are bound to the HLA molecule with different avidities and that there is a defect in the structure of alloantigens on leukaemic cells. Such a defect in structure could hamper hypothetical host defence mechanisms and might explain the difficulties in serological histocompatibility testing of leukaemic lymphocytes (Seigler *et al.*, 1971), since the association with β_2-microglobulin seems to be a prerequisite for a functionally significant binding of alloantisera to alloantigens (Osberg *et al.*, 1975; Welsh *et al.*, 1977).

If the two chain model is sustained, our data indicate that some β_2-microglobulin-associated molecules are lost from the leukaemic cell membrane, or are found in decreased amounts. A decrease in the 'site density' of (tumour-specific?) surface antigens (assuming an unchanged cell surface area (Tivey, Li & Osgood, 1951)) will also favour the proliferation of the malignant cell clone in competition with the host immune system.

The lack of correlation between cell-associated and serum β_2-microglobulin is not surprising, bearing in mind results from *in vitro* studies (Nilsson, Evrin & Welch, 1974) and the influence of kidney function on the serum concentration of β_2-microglobulin (Bernier, Cohen & Conrad, 1968; Johansson & Ranskov, 1972; Wibell, Evrin & Berggard, 1973).

By size and affinity chromatography, we found no evidence for an association of β_2-microglobulin and HLA antigens in the serum from leukaemic patients. This is in accordance with *in vitro* studies on the catabolism of the β_2-microglobulin–HLA complex (Cresswell *et al.*, 1974).

The finding of a selective reduction of non-Con A β_2-microglobulin on leukaemic human cells, together with an increase in the serum concentration of β_2-microglobulin, point towards a defect in the quantitative or qualitative expression of β_2-microglobulin-associated membrane structures in human leukaemia, and increases the need for a complete serological, biochemical and functional characterization of these molecules.

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