Common All Associated Antigen from Cell Surface and Serum: Molecular Properties and Clinical Relevance*

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Introduction

Detection of distinct cell surface markers has brought up remarkable improvements not only in diagnosis and classification of leukemic diseases, but also has gained immediate clinical relevance concerning therapeutic aspects. Especially, such markers may give information about the antigenic phenotype reflecting the origin of distinct leukemic cells from different stem cell compartments and their degree of maturation.

Recently a cell surface antigen has been detected, which so far seems to be highly specific for common ALL (cALL) cells. Interestingly, in some cases this antigen has also been found on T-ALL cells and on cells appearing in blastic crises of CML [7]. Nevertheless, the detection of this common ALL associated antigen (Ag cALL) has been proved to be useful in clinical practice especially in combination with other routinely applied tests for cell surface markers, e.g. E-rosetting, immunoglobulines and C-receptors. We here describe the solubilization and partial physico-chemical characterization of the cALL associated surface antigen. Furthermore, a glycoprotein exhibiting the cALL antigenic specificity could be isolated from the sera of two patients in active relapses of common ALL.

Material and Methods

Chemicals

Phosphate buffered saline (PBS, 10 mM phosphate buffer, pH 7,2, 0,14 M NaCl, 0,02% NaN_3). Sodium deoxycholate was purchased from Merck, Darmstadt. Ficoll and Sepharose CL-6B were products of Pharmacia, Uppsala. Ultrogel AcA 34 was from LKB, Bromma. The reference proteins, myoglobin (purest, from horse), ovalbumin (crystallized two times, from chicken egg) were from Serva, Heidelberg, and bovine serum albumin was from Behring-

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werke, Marburg. Blue dextran 2000 was from Pharmacia, Uppsala, and DNP-α-alanine from Serva, Heidelberg. Aldolase (from rabbit muscle) was from Boehringer Mannheim. β-Lactoglobin (purest, from milk) was from Behringwerke, Marburg.

Cells and Antisera

Peripheral blood lymphocytes (PBL) and bone marrow cells (BMC) were prepared from the following groups of donors: healthy persons, patients suffering from different infectious diseases, non-leukemic hematological disorders, and various types of leukemia, the latter classified according to standard clinical, hematological and immunological criteria [9,11].

In analytical experiments Ficoll isopaque purified cALL-cells of patient T.I. were used as standard targets. Batches of these cells, labelled to 98% by anti-cALL-serum (A-cALLS), were preserved by freezing as described earlier [8]. A-cALLS were raised in rabbits according to Greaves et al. [4] and heat inactivated at 56°C for 30 min. Standard absorptions of A-cALLS with AB-erythrocytes, human AB-serum crosslinked by glutardialdehyde, tonsillar lymphocytes, normal bone marrow cells, cells of acute myelocytic leukemia and normal peripheral blood cells were performed as described in detail recently [8]. Indirect immunofluorescence was used to check for completeness of absorption. The IgG-fraction of the so absorbed A-cALLS was prepared by ammonium sulfate precipitation and subsequent anionic exchange chromatography on DEAE-52 [6].

The specificity of the A-cALLS after the final absorption step is ascertained by using different target cells.

Tetra-ethyl-rhodamine (TRITC) conjugated goat anti-rabbit globulin (TRITC/GARG) was from Nordic Immunologic Laboratories/Tilburg.

Preparation and Solubilization of cALL Cell Membranes

Human ALL-cells (10^9) were homogenized in 20 ml 0,02M Tris, pH 8,0, 0.25M sucrose at 4°C using a Potter Elvejhem homogenizer (3 × 1 min at 1400 rpm). The homogenate was sonicated for 3 × 1 min (MSE sonicator) at 18 microns/min while kept on ice. The sample was then centrifuged for 20 min at 10000 g and the supernatant chromatographed on a Sepharose Cl-6B column (100 × 2,5 cm; flow rate: 40 ml/hr, fraction volume: 10 ml). The material eluted with the void volume containing the cALL plasma membranes was solubilized as follows: 10 ml samples were dialysed step-wise first against 2% (w/v) sodium deoxycholate (6 hr at room temperature) and then against 0,2% sodium deoxycholate (12 hr at 4°C). The dialysed samples were concentrated by ultrafiltration (Amicon PM 10) to a volume of 2,5 ml. The solubilized material was chromatographed on an Ultrogel LKB AcA 34 column (1,0 × 90 cm; flow rate: 15 ml/hr, fraction volume: 3,0 ml), equilibrated with 0,14M NaCl, 10 mM Tris, pH 8,0, 0,02% NaN_3 and 0,2% sodium deoxycholate.

Preparative and Analytical Chromatography of the Ag cALL from Serum of cALL Patients

For analytical chromatography of Ag cALL the 20–50% ammonium sulfate precipitate of 20 ml serum of a cALL patient was dissolved in 20 ml PBS and subjected to gel filtration on AcA 34 (90×5 cm; buffer PBS; flow rate: 60 ml per hr. fraction volume: 5 ml). 30 ml of the combined fractions containing the Ag cALL activity (peak II, Fig. 5) were further purified on agarose bound lentil lectin columns (1,6 mg protein/ml agarose, column size 8,0×0,9 cm, flow rate: 16 ml/hr, fraction volume: 10 ml). Before eluting the antigen with 30 ml 0,2 M α-Methyl-D-Mannoside the column was washed with 50 ml PBS. Analytical gel filtration was performed on an AcA 34 column (90×2,5 cm; buffer: PBS; flow rate: 16 ml/hr). The apparent molecular weight was calculated from Kd versus logarithm of molecular weight of the reference proteins (Fig. 6).

Testsystem

Testing of the absorbing capacity of Ag cALL solubilized from plasma membranes of serum of either cALL patients, healthy control persons or patients suffering from different other leukemic diseases was performed as described in detail recently [8].

The chromatographed solubilized fractions of the plasma cell membranes and of the serum preparations were dialysed against distilled water for 12 hours and lyophilized subsequently. The single fractions were then dissolved in 100 µl PBS. For absorption 20 µl samples each were mixed with 2 µl A-cALLS (final dilution 1 : 11). The mixtures were first incubated for 20 minutes at room temperature and then for 5 hours in the cold. Insoluble material was removed by centrifugation. The supernatants were tested for residual Ag-cALL antibodies by indirect immunofluorescence using (TRITC/GARG) with standard cALL-cells as targets as described previously in detail [8]. The absorbing capacity was calculated according to the formula:

$$\% \text{ absorbed activity} = 100 - \frac{\% \text{ stained cells after absorption}}{\% \text{ stained cells before absorption}} \times 100$$

Unspecific binding was prevented by performing the indirect immunofluorescence assay in the presence of 0,2% sodium azid and Heparin (500 U/ml) 20 µl/ml [2]. Applying this technique the background of unspecific staining was less than 0,2%.

Polyacrylamid-Gel-Electrophoresis (PAGE)

The PAGE in slab gel was performed according to Maizel [10]. The samples (4 µg protein) were applied to 10% polyacrylamid gels and subjected to electrophoresis (35 m A, 3 hours at room temperature). The protein was stained by coomassie blue. The following proteins were used for calibration: Aldolase, Ovalbumin, β-Lactoglobulin and Myoglobin.

Results

Reactivity of Different Human Target Cells to Rabbit A-cALLS

The suitably absorbed A-cALLS showed a high specificity for cALL-cells (Table 1). No binding has been observed to cells of other leukemic diseases and non-leukemic hematological disorders with two exceptions: 1. Ag cALL positive cells were found in bone marrow and, in further course of the disease also in PBL of a two year old boy suffering from CML. 2. In a new born male patient with Trisomie 21 and leukemoid reaction 60% of peripheral leuko-

Table 1. Binding of standard A-cALLS[a] to various human blood cells

Cell type	Number of cases	Positive reaction
1. Normal cells		
peripheral blood lymphocytes	4	0[b]
bone marrow cells	4	0
2. Tumor cells		
common ALL	32	30[c]
T-ALL	7	0
B-ALL	1	0
acute myelocytic leukemia (AML)	9	0
chronic myelocytic leukemia (CML)	7	1
chronic lymphatic leukemia (CLL)	4	0
juvenile chronic myelocytic leukemia (JCML)	2	0
acute monocytic leukemia (AMOL)	2	0
acute myelocytic-monocytic leukemia (AMML)	1	0
osteomyelofibrosis/sclerosis (OMF/S)	2	0
3. Leukemoid reaction		
trisomy 21	1	1
4. Infectious diseases		
mononucleosis	3	0
measles	1	0
tonsillitis	2	0
pneumonia	1	0
pertussis	2	0
5. Non-leukemic hematological diseases		
panmyelopathy	3	0
idiopathic thrombocytopenias	2	0
thalassemia	1	0

[a] Standard absorption was performed with: AB-erythrocytes, glutardialdehyde fixed human AB-serum, tonsillar lymphocytes, normal bone marrow cells, AML cells and peripheral blood lymphocytes.

[b] 0 means no more than 0,2% of positive cells were found. This is in the range of the background found with normal rabbit serum used as control in all immunofluorescence assays.

[c] In our cases of positive reactions at least 30% of the mononuclear cells were stained with the standard absorbed A-cALLS. In leukemias the percentage of the so labelled cells was in most cases close to that of cells identified as leukemic cells by standard morphological, cytological and cytochemical methods

cytes had expressed Ag cALL. Four weeks later we detected only 4% of Ag cALL positive cells in the peripheral blood of this boy and after another two weeks all Ag cALL bearing cells had disappeared.

Solubilization and Chromatography of Membrane Ag cALL

In pilot experiments it was found that sodium deoxycholate, up to a final concentration of 2% (w/v) did not influence the antigenicity of the structure under study to any significant extent. Therefore we used this procedure as a standard method to solubilize cALL plasma membranes. The solubilized membrane antigens were chromatographed on LKB AcA 34 in the presence of 0,2% sodium deoxycholate and tested for their ability to absorb the antibodies against Ag cALL out of A-cALLS. As shown in Fig. 1, the antigenic activity of Ag cALL was detected in three peaks: The bulk of the antigenic activity was found with fractions 37–42 (peak III). A small amount of this antigenicity eluted with fractions 31–33 (peak II). In addition there was antigenic activity associated with unsolubilized material, which eluted with the void volume. By comparison with reference proteins (see legend Fig. 2), the antigenic molecules of peak II and III were estimated to have an apparent molecular weight of 110000 (peak II) and 55000 (peak III) respectively (Fig. 2).

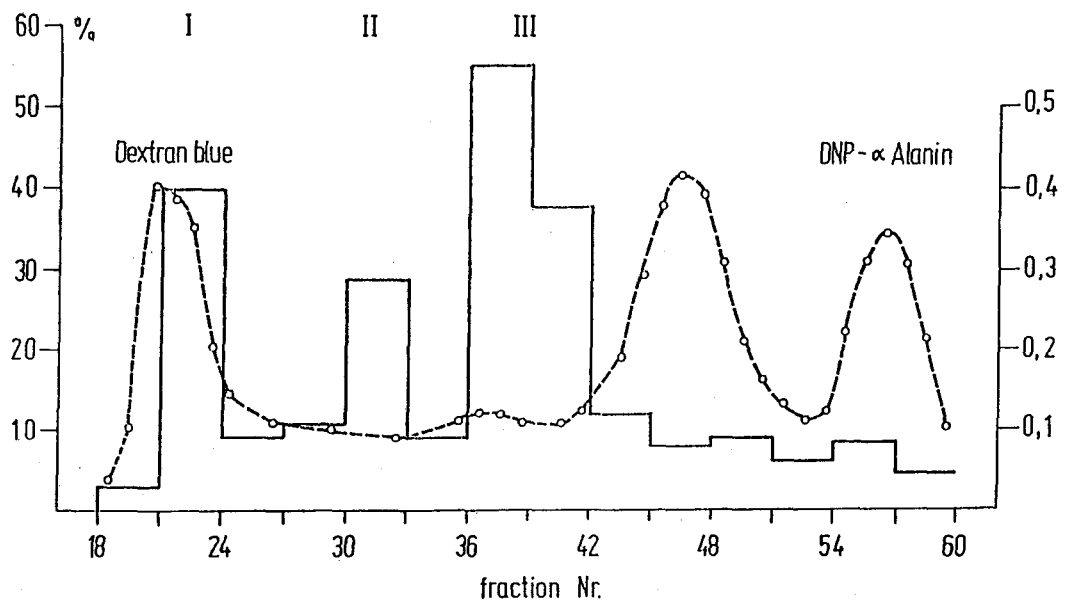

Fig. 1. Analytic absorptions of standard absorbed A-cALLS with solubilized and chromatographed common ALL membrane fractions. The elution volume was collected in 42 fractions (18–60). Every three of these fractions were pooled, lyophilized and resuspended in 100 µl HBSS. For absorption 20 µl of these fractions were incubated with 3 µl standard absorbed A-cALLS (final dilution 1:8). Absorbed antibodies ○——○ E 280 nm

Absorption of A-cALLS with Normal and cALL-Serum

Sera of two cALL patients having Ag cALL positive cells in their peripheral blood were taken for partial characterization of the serum Ag cALL. As may

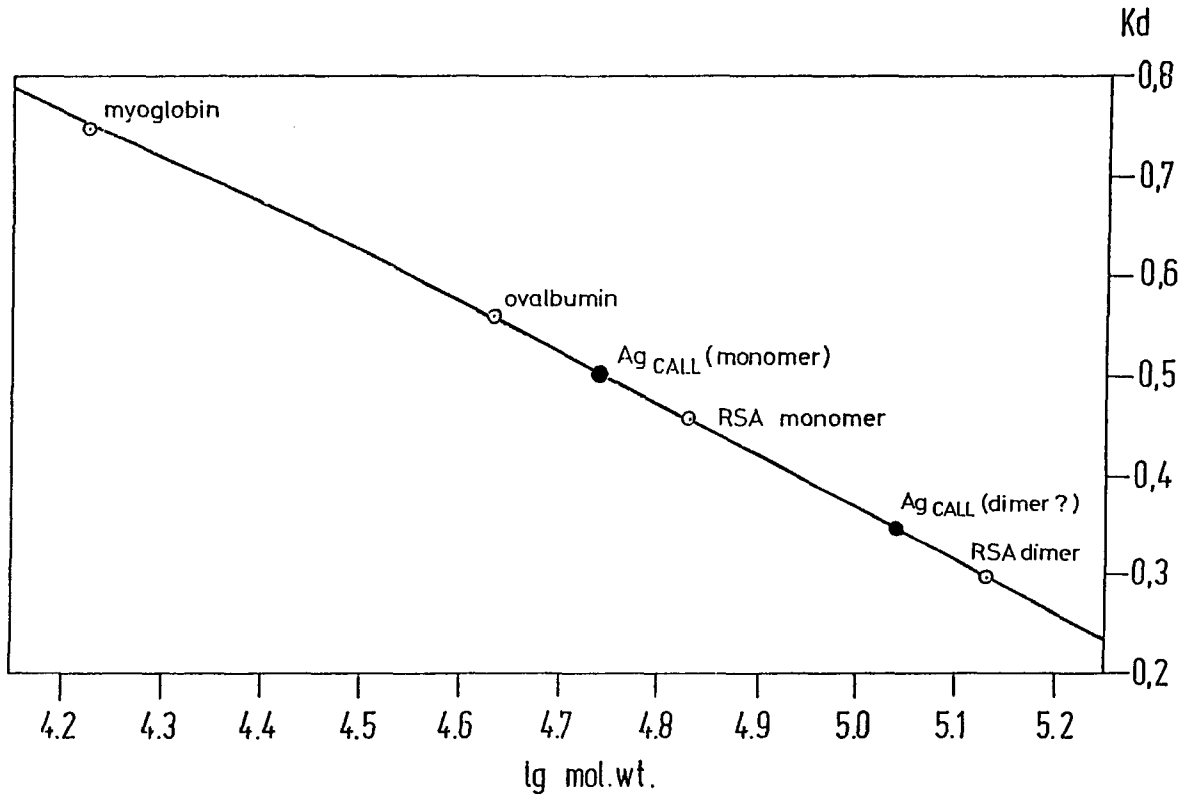

Fig. 2. Calculation of the apparent molecular weight of membran Ag cALL on an AcA 34 column equilibrated with 0.2%; sodium deoxycholate BSA = bovine serum albumin. The molecular weight was calculated from the plot of Kd versus lg molecular weight

be seen from Fig. 3 both sera removed cALL specific antibodies out of A-cALLS. No absorbing effect was seen when sera from normal persons as well as from patients with T-ALL, CLL, AML, AMML and CML were used instead.

Ammonium Sulfate Fractionation and Analytical Chromatography of Serum Ag cALL

Most of the serum cALL associated antigenicity could be precipitated at 30–50% ammonium sulfate saturation. A smaller amount was also found to be pre-

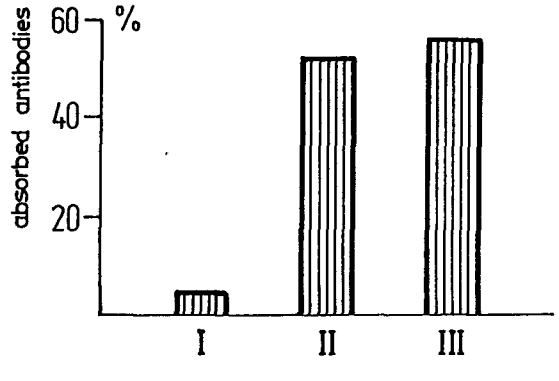

Fig. 3. Analytical absorption of A-cALLS with normal serum and serum of cALL patients. I normal serum; II serum from patient T.F., 2×10^4 cALL cells/µl; III serum from patient T.I., 6×10^4 cALL cells/µl

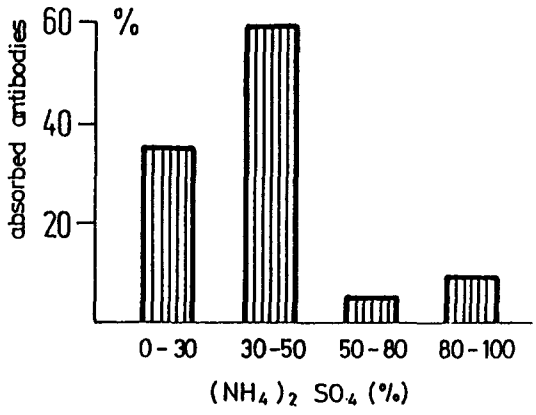

Fig. 4. Fractional precipitation of serum Ag cALL by ammonium sulfate at pH 7,5. 1 ml serum from cALL patients was diluted 1:10. 60 min after adding the desired amount of $(NH_4)_2SO_4$ the sample was centrifuged for 10 min at 12000×g. The sediment was then dissolved in 1 ml PBS and dialyzed for 12 hr against this buffer. 20 µl of the single fractions were used for absorption

cipitated between 0–30% (Fig. 4). By chromatography of the ammonium sulfate precipitated and redissolved cALL-serum fractions on LKB AcA 34 it was found that the elution diagram was characterized by two antigen active peaks (Fig. 5). Peak I eluting with the void volume and peak II between fraction 148–187 corresponding to a molecular weight of 125000 (Fig. 6). After rechromatography of peak II under identical experimental conditions again two antigen active peaks were observed both having the same positions as shown in Fig. 5. The combined fractions of peak II were applied on to an agarose lens culinaris hemagglutinin A column. As may be seen from Fig. 7 the antigen activity bound completely to lens culinaris lectin and could be

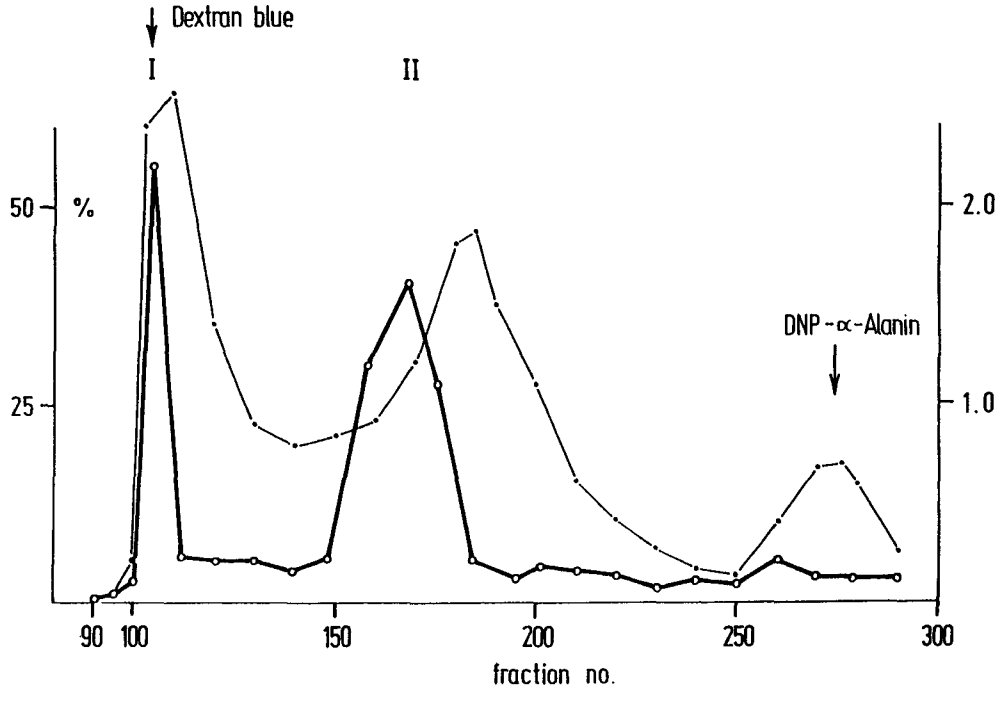

Fig. 5. Chromatography of the 20–50% ammoniumsulfate precipitated Ag cALL from cALL serum on LKB AcA 34; ●——● E 280, ○——○ absorbed antibodies

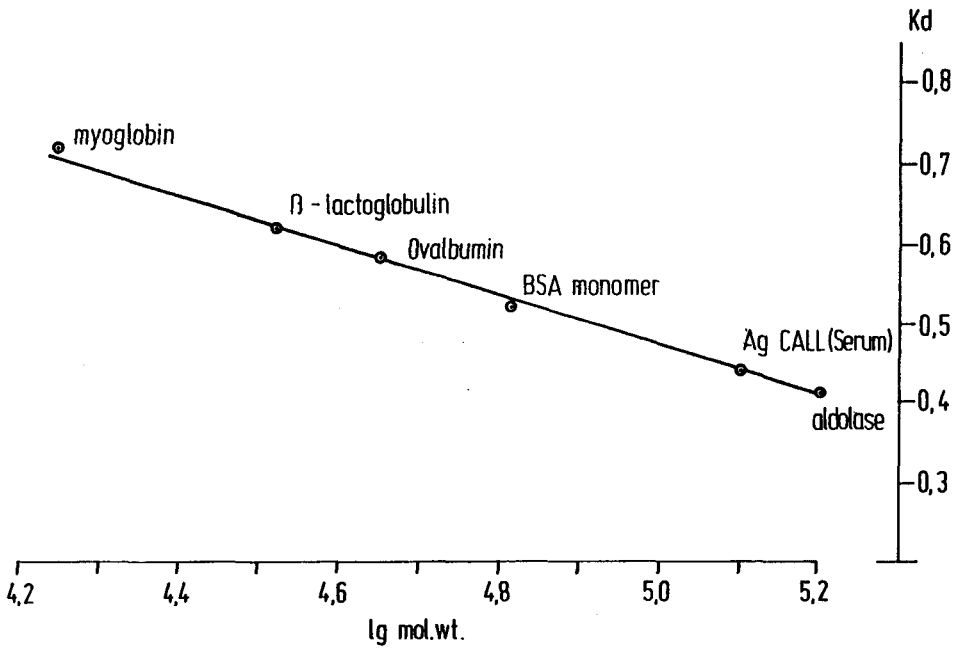

Fig. 6. Calculation of the apparent molecular weight of serum Ag cALL by means of AcA 34 column chromatography. BSA = bovine serum albumin. The molecular weight was calculated from the plot of Kd versus lg molecular weight

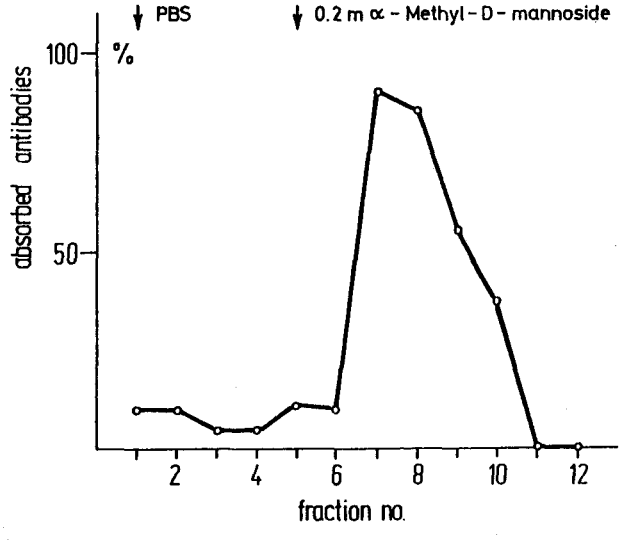

Fig. 7. Affinity chromatography of serum Ag cALL on agarose lens culinaris hemagglutinin A. 30 ml of the combined fractions of Peak II (Fig. 5) after gel chromatography were applied

specifically eluted by 0,2M a α-Methyl-D-Mannoside. The SDS (0,1%) PAGE of this material showed a single major band running at 140000 daltons under unreducing conditions (Fig. 8).

Discussion

Our experiences concerning the appearance of Ag cALL on the surface of leukemic cells and especially on those of cALL are in good agreement with those of Greaves et al. [5] and Rodt et al. [13]. The detection of Ag cALL on blastoid cells from a patient with Trisomie 21 apparently having a leukemoid

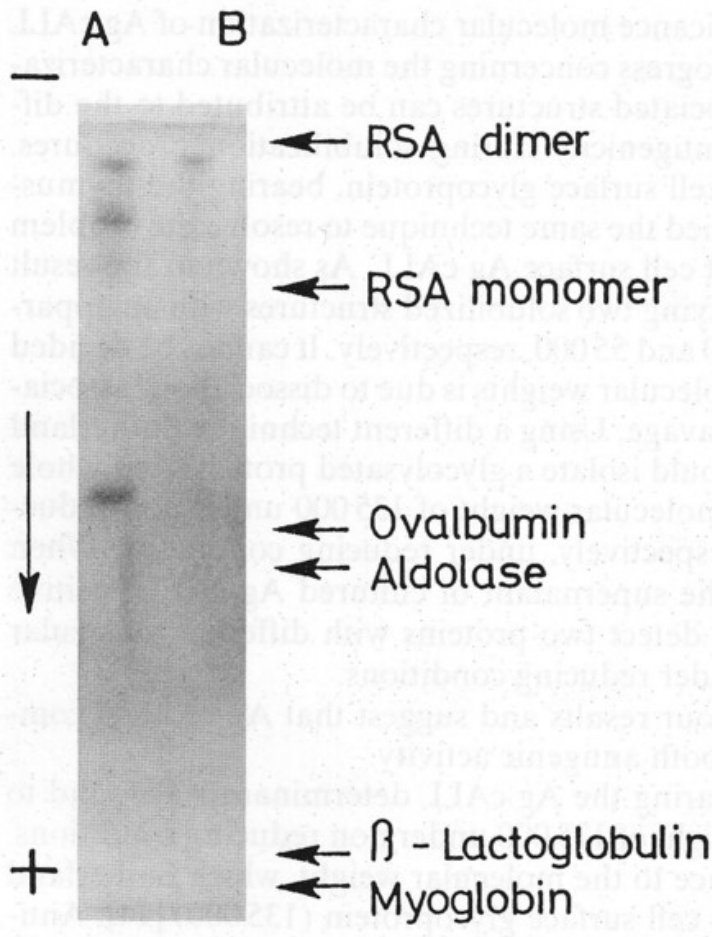

Fig. 8. SDS-Polyacrylamid gel electrophoresis on 10% Slab gels of serum Ag cALL preparations before (left column A) and after (right column B) affinity chromatography on agarose lens hemagglutinin A

reaction suggests that the Ag cALL might be not confined to cALL cells and certain other leukemic diseases. Evidence for this suggestion is also gathered by Roberts et al. [12], demonstrating binding of A-cALLS to rare cells in fetal, neonatal and regenerating bone marrow. Although consequently A-cALLS seems to be not specific for cALL cells, it may be used for clinical purposes with great advantage. This may be demonstrated by the following clinical examples.

On examining the bone marrow of a CML case when first admitted to the clinic, we found no Ag cALL positive cell in peripheral blood (700000 leukocytes/mm^3), but 7% of the bone marrow cells bound A-cALLS. After 6 months the patient developed a blastic crisis. 60 per cent of peripheral blood cells now were found to be Ag cALL positive blast cells. Up to this blast crisis the patient had been treated according to a CML regimen. This was now replaced by an ALL therapy concept, by which the blastic crisis could be controlled within a few days. By this observation it might be concluded that imminant blastic crisis can be detected by frequent monitoring of CML cases with anti-cALLS. Moreover, it should be considered if a combined CML-ALL therapeutic protocol applied at the first appearance of even a few Ag cALL positive cells may prevent blastic crises. In six cases where the clinical, morphological and cytochemical findings were inconclusive, an relapse affecting the CNS could be ascertained by demonstrating Ag cALL positive cells in the CSF even in samples with cell counts as low as 4 cells/mm^3.

Because of its clinical significance molecular characterization of Ag cALL is of great interest. The little progress concerning the molecular characterization of plasma membrane associated structures can be attributed to the difficulties of preserving their antigenicity during solubilization procedures. Recently we isolated another cell surface glycoprotein, bearing the thymus-brain antigen [1]. Now we applied the same technique to resolve the problem of isolating antigenically intact cell surface Ag cALL. As shown in the result section we succeeded in identifying two solubilized structures with an apparent molecular weight of 110000 and 55000, respectively. It cannot be decided whether the 2:1 ratio of the molecular weights is due to dissociation/association events or to enzymatic cleavage. Using a different technique Sutherland (communicated by Greaves) could isolate a glycolysated protein from whole cell extract with an apparent molecular weight of 135000 under non-reducing conditions and 100000, respectively, under reducing conditions. When investigating Ag cALL from the supernatant of cultured Ag cALL positive leukemic cell lines they could detect two proteins with different molecular weight (100000 and 38000) under reducing conditions.

These data are supporting our results and suggest that Ag cALL is composed of two subunits having both antigenic activity.

The serum glycoprotein bearing the Ag cALL determinant was found to have an apparent molecular weight of 125000 under non reducing conditions. This result is in good accordance to the molecular weight, which Sutherland et al. found for the unreduced cell surface glycoprotein (135000) [14]. Antigenic and lectin specificity correspond to the data presented by Brown et al. [3] and Sutherland et al. [14]. Interestingly, in our experiments additional cALL antigen activity was found in the void volume after gel chromatography of serum Ag cALL on AcA 34. The observation that after rechromatography of the combined fractions of peak II (mol. weight 125000) parts of the antigen activity again eluted with the void volume suggests its tendency to associate under the chosen experimental conditions. The apparent molecular weight of the associated molecules of the Ag cALL is presently under study.

The appearance of soluble Ag cALL in the serum indicates shedding or secretion of this material under in vivo condition. These suggestions are supported by the fact that the cALL antigen is found under in vitro conditions in the culture supernatant of native leukemic cells, cell lines derived from leukemic patients and released by the MOLT-4 cell line, which has no detectable cell surface Ag cALL [14].

The binding of nearly equal amounts of anti-Ag cALL antibodies by the sera of the patients (T.I. 54 years old ♀ 60000 leukemic cells/mm^3; T.F. 8 years old ♂, 20000 leukemic cells/mm^3) although differing in their peripheral leukemic cell count by three fold indicates different releasing activity for Ag cALL probably depending on the type and state of the cALL disease. If the serum Ag cALL is common to a vast proportion of acute lymphocytic leukemias the detection of this antigen in the serum might possibly improve diagnosis and therapeutic monitoring of common ALL, especially if it could indicate subclinical disease.

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