A monoclonal antibody VCD-1, directed against the N-terminal intracellular part of the invariant chain (Ii) was used to show, by immunoprecipitation and Western blotting, the unprocessed and processed forms of Ii in chronic lymphocytic leukemic (CLL) cells, in Epstein-Barr virus–transformed normal lymphocytes (EBVL), and in cells of the Raji Burkitt’s lymphoma cell line. Terminal glycosylation and sulphation of Ii in the Golgi apparatus was shown in Raji cells and not in EBVL. CLL lymphocytes contain a higher concentration of p35 Ii than do EBVL or Raji cells.

The role of the Ii chain in these events is believed to be threefold. It blocks the αβ antigen-binding site, thus preventing display of endogenous peptides on class II molecules; it maintains the αβ chain complex in an immunogenically effective conformation; and it targets the αβ chains to an endocytic compartment. Thus, the invariant chain is a potentially important participant in B-cell function, particularly as this involves antigen presentation to CD4+ lymphocytes.

Electrophoretic studies have identified several forms of Ii that differ in charge and mass. These represent the products either of translation from two different AUG initiation codons (giving rise to 33-Kd and 35-Kd proteins) or of alternate splicing of the mRNA (giving 41-Kd and 43-Kd forms). During subcellular passage, glycosylation, and sialylation, limited proteolytic cleavage and sulfation (to form the core protein of a chondroitin sulfate proteoglycan) contribute further to the heterogeneity of Ii chain types that are encountered.

It is known that chronic lymphocytic leukemia (CLL) cells express large amounts of Ii, and it has also been shown that neoplastic cells may show abnormalities in glycosaminoglycan synthesis. For these reasons, we felt that it would be of interest to study the Ii chain synthesis in peripheral blood (PB) lymphocytes from patients with CLL and to compare it with that in Epstein-Barr virus (EBV)–transformed normal PB lymphocytes (EBVL) and neoplastic Raji cells derived from a B lymphoma.

We have developed an IgG2a monoclonal antibody (MoAb), VCD-1, that reacts with an epitope on the N-terminal intracellular region of Ii. In this study, we report on experiments in which we have used VCD-1 to show that processing of Ii does not follow the same pattern in all class II positive cells and that CLL lymphocytes express an unusually large amount of the minor p35 form of Ii.

MATERIALS AND METHODS

Antibodies. VCD-1 MoAb reacts with an intracellular amineterminal epitope on Ii. It was produced in this laboratory by fusion of SP2 myeloma cells with splenocytes from a Balb/c mouse that had been immunized with CLL lymphocytes. Biotinylated VCD-1 was prepared according to the method of Stahl et al. Sheep antimouse Ig antibody and monoclonal mouse anti-horseradish peroxidase were produced in this laboratory.

Cells. The Raji cell line was obtained from American Type Culture Collection (Rockville, MD); the EBVL cell lines were established by transformation of normal PB lymphocytes with EBV from the supernatant of B95-8 cells. CLL cells were PB lymphocytes from patients with CLL and were isolated by Ficoll-Hypaque flotation. Cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics.

Reagents. The following reagents were obtained commercially from the indicated suppliers: formalin-fixed suspension of Staphylococcus aureus (SA), Gibco-BRL (Grand Island, NY); 35S-sodium sulphate, 3H-leucine, Amplify, and Hyperfilm MP, Amersham (Arlington Heights, IL); endoglycosidase-H (endo-H) and long-arm biotin, Sigma (St Louis, MO); Amphotolines, Pharmacia-LKB (Piscataway, NJ); urea, Schwarz-Mann (Orangeburg, NY); Immobilon P (polyvinylidene difluoride membrane), Millipore (Bedford, MA); and avidin/biotinylated peroxidase (ABC) reagent, DAKO (Glostrup, Denmark). Peroxidase-antiperoxidase (PAP) reagent was produced in this laboratory; it contained monoclonal mouse antiperoxidase antibody (ascites at 1/100 dilution) and horseradish peroxidase (Serva, Cape Town, South Africa) at 100 μg/mL of phosphate-buffered saline (PBS).

Preparation of cell lysates. Cells were washed twice in PBS and
CLL lymphocytes. After 20 to 30 minutes on ice, nuclei were pelleted at 1,000g; supernatants were stored at −80°C until use.

**Western blotting.** Cell lysates were electrophoresed in polyacrylamide gels, in the presence of 2% sodium dodecyl sulphate (SDS-PAGE), using the Laemmli buffer system under nonreducing conditions. Blotting and labeling of the blot with VCD-1 was performed essentially according to De Blas and Cherwinski. Proteins were electro-blotted onto Immobilon P at 3.5 V/cm overnight. The membranes were blocked with 5% FCS in Tris-buffered saline, and reacted sequentially with VCD-1 ascites (diluted 1/500 in blocking solution), with sheep antimmunoglobulin Ig antibody (whole sheep serum diluted 1/200), with PAP, and, finally, with diaminobenzidine (DAB) containing 0.03% cobaltous chloride.

**Metabolic labeling of cells with 35S-sulphate.** Sulphate-free medium was prepared by adding minimum essential medium (MEM) amino acid mixture (Flow Laboratories, Irvine, CA) and MEM vitamin mixture (International Scientific Industries, Cary, IL) to the salts of MEM with MgSO4 replaced with 170.8 mg/L MgCl2-6H2O. Cells were washed twice with PBS and suspended at 106 cells/mL in sulphate-free medium with 2% dialyzed FCS. After 30 minutes at 37°C, 50 μCi/mL of 35S-sodium sulphate was added; the cells were incubated overnight, washed, and lysed.

**Pulse-chase labeling with [3H]-leucine.** Cells were washed twice with PBS and suspended, at 2 × 106 cells/mL in leucine-free MEM containing 2% dialysed FCS and incubated for 30 minutes at 37°C. [3H]-leucine was then added (final concentration, 200 μCi/mL), and the cell suspension was incubated at 37°C for 10 minutes more. The culture was then diluted with 15 to 20 volumes of warmed RPMI medium containing leucine and returned to the incubator. Samples of the cell suspension were removed after various chase times, washed twice with cold PBS, and lysed as above.

**Immunoprecipitation.** Formalin-fixed S4 cells were washed twice in PBS containing 0.5% Nonidet P-40 + 2 mmol/L leucine + 0.02% azide (S4 buffer) and resuspended at 10% in S4 buffer containing 1 mg/mL ovalbumin. After 30 minutes on ice, 5 μL of VCD-1 ascites was added per 200 μL S4 suspension. After 1 hour on ice, the S4 was washed twice with S4 buffer. For clearing lysates and for control immunoprecipitations, the S4 was coated similarly with an irrelevant MoAb against β-galactosidase. Lysates were first cleared by incubating for 1 hour on ice, with S4 coated with control antibody. The samples were centrifuged, and the supernatants were immunoprecipitated with S4 coated with VCD-1 for 1 hour. The S4 cells were then washed 5 times with S4 buffer, boiled with SDS-PAGE sample buffer (0.063 mol/L Tris-HCl pH 6.8, 10% wt/vol sucrose, 2.3% wt/vol SDS, and 0.002% bromophenol blue), and centrifuged. The supernatant solutions were loaded onto gels.

**Endo-H treatment.** Washed S4 with bound VCD-1 immunoprecipitate were washed twice more with 10 mL/L Tris-HCl, pH 7.6. The pellets were then suspended in 50 μL of 0.15 mol/L citrate buffer, pH 5.5, containing 0.1 mmol/L PMSF and 1 μL endo-H; control samples were suspended in buffer only. The S4 were incubated in a 37°C water bath for 16 hours and centrifuged. The supernatant was removed, 20 μL of nonreducing sample buffer was added, and, after boiling and centrifuging, the supernant was analyzed by SDS polyacrylamide gel electrophoresis.

**Fluorography.** SDS-polyacrylamide gels were soaked in Amphi-fil for 15 minutes, dried, and fluorographed on Hyperfilm MP.

**Two-dimensional electrophoresis.** Nonequilibrium pH gradient electrophoresis (NEPHGE) followed by SDS-PAGE was performed essentially as described by O’Farrell et al. Briefly, 4-mm diameter rods of 4% acrylamide, 9.2 mol/L urea, 2% Nonidet P-40, and 2% amphotilies, pH 3.5 to 10, were prepared in disposable 1-mL glass pipettes. Immunoprecipitates were solubilized in a solution of 9.5 mol/L urea containing 2% Nonidet P-40, 2% amphotilies, and 5% 2-mercaptoethanol and electrophoresed from the anode at 400 V for 4 hours. The rods were extruded, equilibrated in SDS-PAGE sample buffer, and electrophoresed at right angles on an 11% SDS-slab gel. When unlaabeled proteins were immunoblotted from the slab gel, the Immobion membrane was reacted with biotinylated VCD-1 and ABC.

**RESULTS**

**VCD-1 immunoprecipitates all known monomeric forms of II.** When EBVL were labeled with 3H-leucine, lysed, and immunoprecipitated with VCD-1, two-dimensional electrophoresis and fluorography of the acrylamide gel (Fig 1) showed spots representing the p33, p35, and p41 forms of II as well as the p25 proteolytic cleavage fragment. The most abundant form was that with molecular weight (Mw) 33 Kd (p33); p35, the product of translation from an alternative RNA initiation codon, and p41, which is encoded by mRNA with alternatively spliced exons, were precipitated in lesser amounts.

**CLL lymphocytes express a relatively large amount of the p35 form of II.** In Western blots of EBVL and Raji cell lysates, VCD-1 labeled a prominent band of the major p33 form of II, the 66-Kd dimeric complexed form, and a faint band of M, 35 Kd that could have represented the p35 form or the sialylated, "processed" form of p33. However, in CLL lymphocytes, this higher M, form was as abundant as the major p33 form (Fig 2A). When Western blots were performed with cell lysates of EBVL from 5 different donors (Fig 2B) and of lymphocytes from 5 different CLL patients (Fig 2C), the same pattern was consistently observed. CLL lymphocytes contained more of the 35-Kd species, relative to the 33-Kd species, than do EBVL.

To determine if the more abundant 35-Kd form in CLL cells represented p35 or the sialic acid-derivatized form of p33, we immunoprecipitated cell lysates with VCD-1 and separated the precipitated proteins by NEPHGE and SDS-PAGE in two dimensions. These were then electrophotted and detected with biotinylated VCD-1 and ABC reagent (Fig 3). Clearly, CLL lymphocytes expressed a large amount of the p35 form of II and very little processed p33 (Fig 3B). EBVL (Fig 3A) and Raji cells (Fig 3C) produced only very small amounts of p35 and slightly more of the sialic acid-derivatized protein. Figure 3D shows an immunoblot with VCD-1 of CLL lystate immunoprecipitated with control antibody.

It is processed to form the core protein of significant amounts of chondroitin sulphate proteoglycan (CSPG) in Raji cells but not in EBVL. When Raji cells and EBVL were labeled with 35S-sulphate, lysed, immunoprecipitated with VCD-1, and analyzed by SDS-PAGE, the results, shown in Fig 4, were strikingly different. No CSPG could be precipitated from EBVL (lane 4), whereas Raji cells had synthesized a polydisperse "smudge" of labeled protein...
ranging in M, from approximately 63 to 97 Kd (lane 3). The labeled, unprecipitated cell lysates were included in the gel (lane 1, Raji; lane 2, EBVL) to show that proteoglycans, although of different M, s, were produced by EBVL, but these did not bind to VCD-1. CLL cells produced no detectable CSPG (results not shown).

**DISCUSSION**

The results of these studies have shown that the MoAb VCD-1 precipitated the p33, p35, and p41 forms of the class II invariant chain and their sulphated and sialylated derivatives. Thus, the antibody could be used to estimate relative abundances of the different li species in lymphocyte populations representative of the transformed or neoplastic B-cell phenotype. It is from this application that two observations of interest emerged.

First, PB lymphocytes from patients with CLL were consistently found to contain larger amounts of the p35 form of the li chain relative to the p33 species. On the other hand, in the case of EBV-transformed normal B cells or Raji cells, a very faint 35-Kd band was seen, and p33 was the predominant species. Two-dimensional electrophoresis confirmed the identity of the 35-Kd band in CLL as p35.

Although CLL lymphocytes are known to express increased levels of li, 16 this is, to the best of our knowledge, the
first report of the excessive accumulation in these cells of the Ii species whose translation is initiated from an AUG codon that is upstream from the p33 start codon. The abnormal p35:p33 ratios seen in all of the CLL cells that we examined suggests that this is a characteristic feature of this neoplastic disorder and, as such, merits further study. The fact that both p33 and p35 species are present, but in abnormal relative amounts, raises the possibility that mechanisms that control translation, and that have hitherto been characterized only in prokaryotic cells,27,28 may be deranged in CLL. The excessive accumulation of p35 in CLL cells is of interest because this molecule is known to have a strong N-terminal ER-retention signal10 and to form mixed trimers with p33.29 Therefore, in CLL cells it seems probable that both p35 and...
p33 are retained in the ER without passing through the Golgi apparatus. This is consistent with our observation that glycosylation and sialylation of Ii chains was barely detectable in CLL cells.

In an elegant series of transfective experiments, Clements et al. were able to correlate Ii chain expression with tumorigenicity of Sa1 murine sarcoma cells. They suggested that overexpressed Ii chains blocked class I1 peptide-binding sites and so prevented presentation, via the class I route, of endogenous tumour-associated peptides. These observations, considered in conjunction with our findings, suggest that excessive Ii chain accumulation in CLL cells may contribute to the expression of the neoplastic phenotype by interfering with effective immune surveillance. Indirect support for this notion is to be found in a report by Sekaly et al. that showed class I1 molecules are able to present endogenous peptides in the absence of invariant chain.

Second, we have shown that Raji cells, derived originally from a malignant Burkitt lymphoma, differ from EBVL in that the malignant cells produced a CSPG with Ii as core protein and added endo-H-resistant complex oligosaccharides to their Ii chains, whereas the transformed cells did so to much less of an extent.

The significance of this finding is, as yet, uncertain. It may be related to the neoplastic nature of the cells, because it is known that intercellular glycosaminoglycans from mammalian tumour tissues differ significantly from those of the tissue of origin. Bono et al. have shown that Mₛ of sulphated Ii chains isolated from malignant B cells are generally larger than those purified from normal B cells.

Furthermore, the in vitro killing of K562 target cells by natural killer cells is inhibited by complex carbohydrates; containing α(2 → 6)-sialic acid residues. Thus, the presence of such polysaccharides on the cell surfaces of B-lym-
phoblastoid cells may confer a selective advantage for the survival of neoplastic cells.

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Processing of HLA-class II invariant chain and expression of the p35 form is different in malignant and transformed cells

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