Actin and tubulin of normal and leukaemic lymphocytes

H. ATKINS and P. J. ANDERSON

Department of Biochemistry, University of Ottawa, Health Sciences Centre, 451 Smyth Road, Ottawa, Ont.
K1H 8M5, Canada

(Received 16 June 1982/Accepted 24 August 1982)

Double labelling and the isolation of actin- and tubulin-derived peptides were used to determine the amounts of these proteins in peripheral lymphocytes from normal donors and from patients with chronic lymphocytic leukaemia. As a precaution against proteolysis, samples were boiled before assay. The actin content of chronic-lymphocytic-leukaemia (CLL) lymphocytes was $8.1 \pm 2.1\%$ of total protein, which was lower ($P < 0.05$) than the amount ($12.8 \pm 3.0\%$) of actin found in normal lymphocytes. The tubulin content of CLL lymphocytes was $4.4 \pm 1.5\%$ of total protein, which was also significantly less ($P < 0.05$) than that of normal lymphocytes, which was found to be $6.1 \pm 1.1\%$.

Alterations in actin structure (Leavitt & Kakunaga, 1980), expression (Leavitt *et al.*, 1980) and in the number and distribution of the helical microfilaments formed from this protein (Pollack *et al.*, 1975; Kopelovich *et al.*, 1977; Bachvaroff *et al.*, 1980) have been shown to accompany cellular transformation in a variety of cell types. Another component of the cytoskeleton, microtubules, formed from the protein tubulin, have also been shown to exhibit altered organization in transformed cells (Puck, 1977; Yahara & Kakimoto-Sameshima, 1978).

Chronic lymphocytic leukaemia is a disease characterized by an accumulation of clonal lymphocytes exhibiting a variety of functional abnormalities associated with properties of the cytoskeleton such as motility (Craig *et al.*, 1976), cap formation (Zucker-Franklin *et al.*, 1979) and susceptibility to certain drugs (Thompson *et al.*, 1975). For this reason, attempts have been made to measure the amounts of actin (Stark *et al.*, 1982) and tubulin (Liebes *et al.*, 1980) in normal and CLL lymphocytes. However, difficulties with the methods used in these studies have been pointed out. Tubulin was estimated by colchicine binding. This technique measures only soluble tubulin and the colchicine-binding site of tubulin is labile (Hiller & Weber, 1978; Bulinski *et al.*, 1980). Actin was measured by densitometry of electrophoretically separated proteins. This method requires that actin

be separated from all other proteins and that all proteins be fixed and stained equally. This is unlikely under the conditions used (Bray & Thomas, 1975). In addition, problems arising from rapid proteolysis in blood-cell preparations (Amrein & Stossel, 1980) may lead to difficulties in measuring the amount of individual proteins in such preparations.

For these reasons, it was decided to determine the amount of actin and tubulin in normal and CLL lymphocytes by a method which allows the accurate measurement of each of these proteins under conditions which overcome problems associated with other methods and by which effects due to proteolysis are eliminated. This method involves the use of double radioisotope labelling and peptide quantification to determine the amount of individual proteins in mixtures. Applications to the measurement of actin (Anderson, 1979*a*) and tubulin (Anderson, 1979*b*) have been described. In the present application, both proteins are measured in the same sample of lymphocytes and the amounts of the proteins in normal and CLL lymphocytes are compared.

Experimental

Materials

Actin from rabbit muscle and tubulin from calf brain were prepared as previously described (Anderson, 1979*a,b*). Iodo[2-¹⁴C]acetic acid was obtained from Amersham/Searle and diluted with carrier to a specific radioactivity of 2 mCi/mmol before use. Iodo[2-³H]acetic acid was obtained from New England Nuclear Corp. and diluted to a specific radioactivity of 100 mCi/mmol before use.

Abbreviations used: CLL lymphocytes, chronic-lymphocytic-leukaemia lymphocytes; SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; CmCys, carboxymethylcysteine.

Lymphocytes were prepared from healthy volunteers and from patients with chronic lymphocyte leukaemia after obtaining informed consent. About 80 ml of blood from healthy volunteers or 20 ml of blood from leukaemic patients was routinely drawn into a heparinized syringe and incubated for 1 h at 37°C to allow sedimentation of erythrocytes. The leukocyte-rich plasma was decanted from the erythrocytes and centrifuged at 2000 *g* for 10 min. Purified mononuclear cells were obtained by centrifugation of the sedimented material for 3 min at 2000 *g* on a Ficoll–Paque (Pharmacia Fine Chemicals) discontinuous gradient. The layer at the boundary was withdrawn and centrifuged at 3000 *g* for 2 min. The precipitate was suspended in Lymphocyte Separator reagent (a carbonyl iron suspension from Technicon Corp.) and incubated at 37°C for 30 min. Monocytes and granulocytes were then removed by using a magnet and the lymphocytes remaining in the suspension were sedimented by centrifugation at 3000 *g* for 2 min. The lymphocyte preparations, usually containing between 10^8 and 2×10^8 cells, were washed with RPMI medium (Moore *et al.*, 1967) without glutamine or serum and stored for not more than 2 months at –80°C before use. Microscopic examination of Wright-stained smears of lymphocytes prepared in this way indicated less than 5% monocyte contamination and negligible amounts of platelets.

Sample preparation

In the early stages of these experiments, electrophoretic examination of lymphocyte proteins indicated that rapid degradation of protein occurred in both normal and CLL lymphocyte preparations. The amount of degradation was variable from preparation to preparation, but appeared to occur to a similar extent in both normal and CLL lymphocytes. Large amounts of low-molecular-weight material were observed when cells were treated with acetone and chemically modified without a preliminary heat treatment of samples. Such degradation was prevented by thawing the frozen cell preparations by placing them in a boiling-water bath for 2 min. The heat-treated material was then cooled on ice and washed with 20 ml of cold acetone three times. The resulting acetone-dried powders were then dried under vacuum. Samples (2–4 mg) of the acetone-dried powders were then chemically modified with iodo[2-³H]acetic acid, dialysed against water and freeze-dried as previously described (Anderson, 1976). Samples of purified rabbit muscle actin and calf brain tubulin were treated in the same way for use as protein standards. Additionally, purified actin and tubulins to serve as internal standards were treated identically except for the use of iodo[2-¹⁴C]acetic acid in the radiolabelling.

Typically, the freeze-dried ³H-labelled samples had a specific radioactivity of approx. 9×10^6 c.p.m./mg, whereas the ¹⁴C-labelled internal standards had a specific radioactivity of the order of 3×10^5 c.p.m./mg.

Actin and tubulin determinations

Actin and tubulin contents of lymphocytes were determined as previously described (Anderson, 1979a,b) except for the following modifications. Known amounts of both ¹⁴C-labelled actin and ¹⁴C-labelled tubulin were added to each sample before electrophoretic separation on SDS/polyacrylamide gels. The location of actin and tubulin in the gels was simplified by the addition of dansylated actin and tubulin (Gray, 1972) to the samples before electrophoresis. The proteins were located after electrophoresis by using a u.v. lamp and the bands corresponding to actin and tubulin were cut out with a razor blade. Material was extracted with 70% (v/v) formic acid overnight. After removal of formic acid under vacuum, residual SDS was removed from the extract by washing with ice-cold 10% (w/v) trichloroacetic acid followed by three washes with cold acetone. After removal of residual acetone under vacuum, the extracted material was suspended in 0.2 ml of 0.5% NH₄HCO₃. Peptide generation and isolations were then carried out as previously described for actin (Anderson, 1979a) and tubulin (Anderson, 1979b) and the ³H/¹⁴C ratios of isolated peptides were used to calculate amounts of actin and tubulin. For actin determination, the ³H/¹⁴C ratios of material co-purifying with two muscle-actin-derived chymotryptic peptides, Lys-CmCys-Asp-Val-Asp-Ile-Arg-Lys-Asp-Leu-Tyr and Arg-Lys-CmCys-Phe on pH 6.5 and pH 2.1 paper electrophoresis were used. These peptides account for Cys-284 and Cys-373 of muscle actin (Collins & Elzinga, 1975). All known actins give rise to peptides co-purifying with them (Vanderkerchove & Weber, 1978). The ³H/¹⁴C ratios of material co-purifying with two peptides produced from tubulin by digestion with elastase were used for the calculation of tubulin content. These were Asp-Trp-CmCys-Pro-Thr, containing Cys-347 of α -tubulin (Ponstingl *et al.*, 1981), and Val-CmCys-Asp-Ile-Pro-Pro-Arg-Gly-Leu, containing Cys-354 from β -tubulin (Krauh *et al.*, 1981) of porcine brain. The presence of these peptides in tubulin from chicken embryo brain (Valenzuela *et al.*, 1981) indicates they are from regions of high homology. Protein was quantified by dissolving weighed amounts of modified lymphocyte proteins in 70% formic acid and measuring the amount of protein in 20 μ l portions containing approx. 50 μ g of material by the method of Lowry *et al.* (1951), with bovine serum albumin dissolved in 70% formic acid as the standard.

Fig. 1. Distribution of radioactivity in SDS/polyacrylamide gels after electrophoresis

[^{14}C]Carboxymethylated actin and tubulin were mixed with [^3H]carboxymethylated proteins of lymphocytes prepared from normal donors and chronic-lymphocytic-leukaemia patients. Lower-molecular-weight material exhibits increased migration to the right in this electrophoresis system. The radioactivity eluted from 1 mm slices in 70% formic acid was determined by liquid-scintillation counting. $\cdots$, [^{14}C] radioactivity of tubulin (low mobility) and actin (high mobility); —, [^3H] radioactivity when proteins of a normal lymphocyte preparation were [^3H]carboxymethylated; ----, [^3H] radioactivity when proteins of a CLL-lymphocyte preparation were [^3H]carboxymethylated.

Results

Fig. 1 shows the distribution of radioactivity in SDS/polyacrylamide gels obtained from one normal donor and from one chronic-lymphocytic-leukaemia patient. Similar amounts of [^3H]carboxymethylated proteins of normal or CLL lymphocytes were mixed with [^{14}C]carboxymethylated actin and tubulin and separated by electrophoresis. This illustrates the difference in protein-labelling patterns between normal- and CLL-lymphocyte proteins. Typically, the region corresponding in mobility to actin contained the largest peak of radioactivity when the proteins of the lymphocytes of normal donors were examined. The pattern obtained for proteins from the lymphocytes of chronic-lympho-

Fig. 2. Actin and tubulin contents of individual lymphocyte preparations

Double labelling and peptide isolation were used to estimate the amount of actin and tubulin. [^3H]/[^{14}C] ratios of two actin- and two tubulin-specific peptides generated from material co-migrating with these proteins on SDS/polyacrylamide gels were used to calculate the amount of actin and tubulin in lymphocyte preparations as described in the text. Horizontal bars indicate the mean values for actin and tubulin in normal (NRM) and CLL lymphocytes (CLL).

cytic-leukaemia patients was more variable, but from Fig. 1 the most striking feature was a greatly diminished amount of radioactivity co-migrating with actin on the SDS/polyacrylamide gels. Another feature illustrated in Fig. 1 is that the proteins of CLL lymphocytes contained less radioactivity co-migrating with tubulin on polyacrylamide gels than the proteins of normal lymphocytes. However, this difference was not as great as the difference in the region corresponding to actin mobility.

There was no evidence of a difference in protein concentration between normal and CLL lymphocytes. In both cases 10^8 cells contained 3.5 mg of protein. Therefore the amount of actin and tubulin in these preparations expressed as percentage (w/w) of total protein is directly related to the actin and tubulin content per cell.

The amounts of actin and tubulin in individual lymphocyte preparations are given in Fig. 2. These values were calculated from the [^3H]/[^{14}C] ratios obtained when peptides were generated from the

Table 1. *Actin and tubulin contents of normal and CLL lymphocytes*

Results are expressed as percentage of total protein $\pm$ s.d. Determinations were made on lymphocytes from eight CLL patients and seven normal donors.

Lymphocyte type	Proportion of total protein (%)	
	Actin	Tubulin
Normal	12.8 ± 3.0	6.1 ± 1.1
CLL	8.1 ± 2.1	4.4 ± 1.5

material co-migrating with actin or tubulin on SDS/polyacrylamide-gel electrophoresis and purified by paper electrophoresis at pH 6.5 and pH 2.1. The mean amounts of actin and tubulin in normal and CLL lymphocytes, expressed as (w/w) percentages of total protein, were calculated from the data shown in Fig. 2 and are given in Table 1. Both actin and tubulin are decreased in CLL lymphocytes compared with normal lymphocytes ($P < 0.05$).

Discussion

Normal lymphocytes have been reported to contain $8.4 \pm 2.1\%$ (w/w) actin compared with $4.3 \pm 1.5\%$ actin in CLL lymphocytes (as percentages of total protein) when actin was determined by densitometry of SDS/polyacrylamide gels (Stark *et al.*, 1982). When densitometry of isoelectric-focusing gels was used to measure actin content, normal lymphocytes contained $6.6 \pm 1.8\%$ actin compared with $4.3 \pm 1.0\%$ actin in CLL lymphocytes. These results agree with the present conclusion that actin as a percentage of total protein is decreased in CLL lymphocytes. However, the amount of actin measured by densitometry in both types of lymphocytes is considerably less than that reported in the present study. Problems in the use of densitometry of polyacrylamide gels to measure the actin content of cells have been pointed out (Bray & Thomas, 1975). In addition, the occurrence of a significant amount of proteolysis during the assay of a protein may lead to low estimates of the amount of that protein. In the preliminary stages of the present study, lymphocyte preparations were not boiled before treatment with acetone and radiochemical modification. The pattern of radioactivity in SDS/polyacrylamide gels after electrophoresis of lymphocyte preparations which had not been boiled indicated large, but variable, amounts of radioactivity in the low-molecular-weight regions of the gels when either normal or CLL lymphocytes preparations were used, and the amounts of radioactivity migrating in the regions of actin and tubulin mobility were diminished. The patterns obtained by densitometry of lymphocyte proteins separated by

SDS/polyacrylamide-gel electrophoresis indicate the presence of large amounts of low-molecular-weight material (Stark *et al.*, 1982) in the preparations used for actin determinations. The lymphocyte preparations were not boiled before cell disruption and when the actin assays were carried out in the presence of the serine-proteinase inhibitor di-isopropyl fluorophosphate, higher apparent actin contents of lymphocytes were obtained. The discrepancy between that study (Stark *et al.*, 1982) and the present report of the amount of actin in lymphocytes may therefore reflect the occurrence of proteolysis in lymphocyte samples that are not heat-treated before cell disruption. Rapid proteolysis resulting in underestimates of contractile proteins in human leucocytes has been reported (Amrein & Stossel, 1980). In replicating human T-lymphocytes, 16% of [35 S]-methionine radioactivity was incorporated in material co-migrating with actin on SDS/polyacrylamide electrophoresis (Leavitt *et al.*, 1980).

Colchicine binding has been used to measure the amount of tubulin in normal and CLL lymphocytes (Liebes *et al.*, 1980). No significant difference in the tubulin content of normal lymphocytes, in which $1.21 \pm 0.79\%$ of the soluble protein was measured as tubulin, and CLL lymphocytes, in which $1.10 \pm 0.43\%$ of the soluble protein was measured as tubulin, was found. In the present study the tubulin content of normal lymphocytes was found to be $6.1 \pm 1.1\%$ of the total protein, which was significantly higher than the tubulin content of $4.4 \pm 1.5\%$ of total protein found in CLL lymphocytes. Proteolysis in lymphocyte preparations that are not heat-treated may again account for the discrepancy. No measures to prevent proteolysis were taken in the colchicine-binding assays. After cell disruption, preparations were centrifuged for 1 h and incubated at 37°C for at least 1 h in these assays. Under these conditions, extensive proteolysis leading to destruction of the colchicine-binding site and consequent high variability and systematic low estimates of tubulin content might be expected. In another study of the amount of tubulin in human peripheral lymphocytes using a colchicine binding assay, only 0.2% of the protein was estimated to be tubulin (Sherline & Mundy, 1977). We are not aware of any other estimates of the tubulin content of human lymphocytes. However, the tubulin contents of many types of cultured cells have been reported. These values, ranging between 3 and 6% of soluble protein (Hiller & Weber, 1978; Bulinski *et al.*, 1980), and obtained by a variety of methods, are closer to the tubulin contents of lymphocytes determined in the present study than to the values estimated by colchicine binding.

The patients in the present study diagnosed as having chronic lymphocytic leukaemia had lymphocyte counts of more than $10 \times 10^9/\text{litre}$. The normal

subjects all had lymphocyte counts of less than 10×10^9 /litre. Although no attempt was made in this preliminary study to correlate actin and tubulin content with the clinical staging of the disease (Rai *et al.*, 1975), the finding of decreased amounts of these proteins in CLL lymphocytes indicates that future studies of this type would be worthwhile.

The ratio of B- and T-cells within the total lymphocyte population are altered in chronic lymphocytic leukaemia, since the disease typically represents a clonal proliferation of B-cells. The results obtained in the present study may arise from this if T-cells contain much larger amounts of actin and tubulin than do B-cells. Previous studies indicate that this is unlikely. No difference in the actin contents of separated B- and T-cells was observed in normal and CLL lymphocytes (Stark *et al.*, 1982). Normal peripheral lymphocytes and a B-cell-enriched population contained similar amounts of tubulin (Liebes *et al.*, 1980).

In order to further define the role of actin and tubulin in the physiology and pathophysiology of lymphocyte function, studies designed to determine the number and distribution of microfilaments and microtubules in normal and CLL lymphocytes are required. Future studies may indicate whether alterations in these structures, as have been observed in other leukaemias (Bachvaroff *et al.*, 1980), accompany the decreased actin and tubulin contents of CLL lymphocytes reported in the present study.

This work was supported by the Medical Research Council of Canada. We thank Dr. J. Maroun of the Ottawa General Hospital and Dr. J. Bormanis of the Ottawa Civic Hospital for help in obtaining blood from chronic-lymphocytic-leukaemia patients and Mr. Martin White for assistance in the preparation of lymphocytes.

References

- Amrein, P. C. & Stossel, T. P. (1980) *Blood* **56**, 442–447
- Anderson, P. J. (1976) *Biochem. J.* **155**, 297–301
- Anderson, P. J. (1979a) *Biochem. J.* **179**, 425–430
- Anderson, P. J. (1979b) *J. Biol. Chem.* **254**, 2168–2171
- Bachvaroff, R. J., Miller, F. & Rapaport, F. T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4979–4983
- Bray, D. & Thomas, C. (1975) *Biochem. J.* **147**, 221–228
- Bulinski, J. C., Morgan, J. L., Borisy, G. G. & Spooner, B. S. (1980) *Anal. Biochem.* **104**, 432–439
- Collins, J. & Elzinga, M. (1975) *J. Biol. Chem.* **250**, 5915–5920
- Craig, S., Snyderman, R. & Chen, H. J. (1976) *Blood* **48**, 717–729
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–137
- Hiller, G. & Weber, K. (1978) *Cell* **14**, 795–804
- Kopelovich, L., Cohlson, S. & Pollack, R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3019–3022
- Kraus, E., Little, M., Kempf, T., Hofer-Warbinek, R., Ade, W. & Ponstingl, H. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4156–4160
- Leavitt, J. & Kakunaga, T. (1980) *J. Biol. Chem.* **255**, 1650–1661
- Leavitt, J., Leavitt, A. & Attallah, A. M. (1980) *J. Biol. Chem.* **255**, 4984–4987
- Liebes, L. F., Fleit, H., Zucker-Franklin, D. & Silber, R. (1980) *Biochem. Biophys. Acta* **633**, 245–257
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Moore, G. E., Jerner, R. E. & Franklin, H. A. (1967) *J. Am. Med. Assoc.* **199**, 515–524
- Pollack, R., Osborn, M. & Weber, K. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 994–998
- Ponstingl, H., Kraus, E., Little, M. & Kempf, T. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2757–2761
- Puck, T. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4491–4495
- Rai, K. R., Sawitsky, A., Cronkite, E. P., Chanana, A. D., Levy, A. N. & Pasternak, B. S. (1975) *Blood* **46**, 219–234
- Sherline, P. & Mundy, G. R. (1977) *J. Cell Biol.* **74**, 371–376
- Stark, R., Liebes, L. F., Nevrla, D., Conklyn, M. & Silber, R. (1982) *Blood* **59**, 536–541
- Thompson, A. E. R., O'Connor, T. W. E., Vaughan-Smith, S. & Farrel, K. W. (1975) *N. Engl. J. Med.* **293**, 939–940
- Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W. & Cleveland, D. W. (1981) *Nature (London)* **289**, 650–655
- Vanderkerckhove, J. & Weber, K. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1106–1110
- Yahara, I. & Kakimoto-Sameshima, F. (1978) *Cell* **15**, 251–259
- Zucker-Franklin, D., Liebes, L. & Silber, R. (1979) *J. Immunol.* **122**, 97–107