Some Properties of a DNA-unwinding Protein Unique to Lymphocytes from Chronic Lymphocytic Leukemia

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SUMMARY

Lymphocytes from a common human leukemia, chronic lymphocytic leukemia, have a greatly enhanced capability of DNA repair and a concomitantly prolonged survival in vitro after damage to DNA. From these lymphocytes, we isolated and purified a DNA-binding protein with a molecular weight of 24,000. It binds tightly to both ultraviolet light (UV)-irradiated and single-stranded DNA. At 35° it enhances the helix-coil transition of poly[d(A-T)] and the UV-irradiated calf thymus DNA but is inefficient in ordinary native DNA. This protein also facilitates the rate of UV-endonuclease incision of UV DNA but does not induce any nicks by itself. This finding suggests that the protein may be involved in DNA repair by enhancing such activity, and also offers an explanation for our observation of increased DNA repair in chronic lymphocytic leukemia cells. When human metaphase chromosomes are exposed to the protein, it induces marked lengthening of chromatids suggesting that this protein may also act on complex chromosomes.

By quantitative immunochemical determinations, such protein could not be found in lymphocyte extracts of three normal individuals.

INTRODUCTION

CLL⁴ is a common form of human leukemia affecting the older age group. The clinical manifestations of this disease are frequently due to a rising leukemic cell mass with organ infiltration, autoimmunity against red cells and tissues, pancytopenia, and hypogammaglobulinemia. These “immunoincompetent” lymphocytes (8), despite their progressive increase in vivo, do not show significant differences in the mitotic rate or [³H]thymidine labeling when compared with normal lymphocytes (both are in the range of 12 to 15 cells labeled with [³H]thymidine per 10⁶ lymphocytes) (6). Therefore, at least 2 possibilities exist which may explain the increasing cell mass in CLL: (a) CLL cells may have a longer life-span with identical proliferative capacity to normal cells, or (b) the proliferative capacity is slightly higher but the difference is below the limit of detection.

In searching for an answer to the above question, we find that CLL cells show an increased capacity to repair DNA damage induced by UV irradiation or X-ray (10). Compared with irradiated normal cells, irradiated CLL cells (a) incorporate [³H]thymidine more rapidly into their DNA during repair synthesis, (b) excise pyrimidine dimers (the major UV photoproduct) more rapidly and to a greater extent, and (c) rejoin X-ray-induced strand breaks more rapidly. Furthermore, UV-irradiated, [³H]thymidine-labeled CLL cells sustain in tissue culture for longer times than do normal cells (11). These results indicate that the CLL cells “survive” UV-irradiation in vitro better than do normal ones. These observations strongly suggest that the 1st possibility, the theory of longer life-span in CLL lymphocytes, may be true.

A search was made for possible increases in the activities of repair-related endonucleases and polymerases in CLL cells. In repeated experiments, no significant differences could be found between normal and CLL cells (A. T. Huang, unpublished data). In a further attempt to correlate the difference in DNA repair with a basic biochemical abnormality, we investigated proteins that have strong affinity toward DNA. This report describes the simple isolation and some properties observed of a DNA-unwinding protein from CLL cells. The protein not only affects the helix-coil transition of DNA, like gene-32 protein of T4 phage, but also facilitates the activity of an endonuclease related to UV excisional repair from Micrococcus luteus. It cannot be detected immunochemically in lymphocytes from normal individuals.

MATERIALS AND METHODS

Lymphocytes from 4 CLL patients and 3 normal donors were obtained by leukophoresis via an Amino blood cell separator (available and operated regularly at Duke Cancer Research Unit with fully informed consent and with permission for human studies from our institution). With this separator, approximately 10¹⁰ to 10¹¹ cells could readily be removed from patients with CLL who had cell counts of over 100,000/cu mm and about 10⁸ to 10¹⁰ cells from normal donors. Fractions of these cells were some-
times cultured in McCoy’s medium with 15% fetal calf serum at 2 x 10^6 cells/ml and labeled with ^3H-labeled amino acids (New England Nuclear, Boston, Mass.) for 16 to 20 hr so as to obtain a radioactive marker before they were processed. The cells were washed with phosphate-buffered 0.15 M NaCl solution (pH 7.4), and then disrupted with a cell-homogenizing bomb (Parr Instrument Co., New York, N. Y.) at 1500 psi for 30 min in 0.05 M NaCl buffer (0.05 M NaCl, 0.02 M Tris, pH 7.4, 1 mM EDTA, 1 mM mercaptoethanol, and 5% glycerol). The homogenates were repeatedly disrupted sonically [(Heat Systems Cell Disruptor, Plainview, N. Y.) Model W185, large probe, maximum, 1-min. interval] to fragment the DNA during the time the ionic strength was gradually brought to 2 M with solid NaCl. Protein with DNA affinity were thus dissociated from DNA. The viscous suspension was then mixed with 20% Carbowax 4000 to precipitate most of the DNA. After centrifugation, the supernatant was sometimes further treated with pancreatic DNase I (100 μg/ml) to remove residual trace amounts of DNA. The supernatant was centrifuged at 130,000 x g for 60 min to remove particulates and then dialyzed to reduce the NaCl concentration to 0.05 M before DNA-cellulose column chromatography.

Affinity chromatography on DNA-cellulose columns was used to isolate the proteins of interest. Columns were made with DS and SS DNA, according to the method of Alberts and Herrick (2) and Herrick (9). A similar procedure was used to make columns with UV-irradiated native DNA [using the photosensitization method of Lamola and Yamane (14) which yielded a high proportion of pyrimidine dimers]. The crude isolate was passed through a native (DS) column first to remove a variety of nucleases, polymerases, histones, and other proteins. The flow-through material was concentrated before it was applied to a 2nd column made of either SS or UV-irradiated DNA. The 2nd column was then washed and eluted with stepwise gradients using different ionic strengths of NaCl buffer (from 0.05, 0.1, 0.6, 1.0 to 2.0 M). Fractions thus eluted were later concentrated and examined for the DNA-unwinding and nucleolytic activities, and also were analyzed for their homogeneity and estimated molecular weights. Repeat column chromatography was useful for improving the purity of the protein fractions.

At various steps of purification, proteins were examined by (a) 10% SDS-acrylamide gel electrophoresis in phosphate buffer described by Weber and Osborn (20) and (b) Sephade G-75 gel filtration.

UV-specific Endonuclease Assay. The assay (4) was carried out by incubating the enzyme from M. luteus (gift of Carrier and Setlow) with or without 25 μg of purified CLL protein at 37° in a 0.1-ml mixture of 2.5 μg of UV-irradiated[^3H]DNA (average distance between dimers of approximately 24,000 daltons) and 25 μg of native[^3H]-DNA (Escherichia coli) in 0.05 M potassium phosphate buffer (pH 6.5) containing 0.01 M mercaptoethanol and 1 mM EDTA. At various times, aliquots of samples were removed, 0.5 N NaOH was added for 30 min to denature the DNA for column chromatography, and its pH was later readjusted with equimolar HCl. The SS products were then analyzed on Sepharose 4B columns to detect changes in approximate molecular weights according to Elution Volume D. Techniques of alkaline sucrose gradient sedimentation of the final products for DNA molecular weight determination have previously been described (15).

Detection of DNA Endonuclease Nicks, Exonuclease, and RNase Activity on Purified Protein Preparations. The change from a circular form of φX 174 RFI to linear DNA induced by endonuclease incision was measured by the method described by Center et al. (5). Exonuclease assay was performed using the method described by Curtis and Smellie (7). Klee’s (13) and Kunitz’s methods were used for detecting RNase activity on 4 S RNA with RNase A (from bovine pancreas, 30 μg/ml; Sigma Chemical Co., St. Louis, Mo.) as positive controls.

DNA Unwinding and the Stoichiometry of Binding on Nuclease-free Protein Preparations. DNA hyperchromicity changes were measured and continuously recorded with a Gilford spectrophotometer immediately after the purified protein was added, with a protein:DNA weight ratio of 10:1 as described by Alberts and Frey (1). The temperature was maintained at 25° or 35°. SSC (1:10; 0.015 M NaCl:0.0015 M sodium citrate) was used instead of the standard buffer. In the temperature range of our experiments, denaturation of protein and appearance of turbidity do not occur. The stoichiometry of binding between protein and DNA was measured by the inhibition of hypochromic shift of SS DNA after addition of Mg^{2+} as described previously by Sigal et al. (17). The maximum inhibition of hypochromic shift thus measured reached a plateau between protein: DNA weight ratio of 6:1 and 10:1, similar to that found by Sigal et al. with the E. coli DNA-unwinding protein.

Production of Rabbit Antiserum Against Purified Protein Eluted from 1 M Buffer on UV-irradiated DNA-cellulose Column. One mg of the purified protein was emulsified in complete Freund’s adjuvant and injected s.c. into white rabbits for 3 times with an interval of 2 to 3 weeks. The presence of an antibody against the purified protein was checked by Ouchterlony double-diffusion technique (16). Studies for immunochemical identification for such proteins from other patients with CLL, CML, and normal lymphocytes were carried out using the above-described technique. The sensitivity of detection was determined by double immunodiffusion between the serum and serially diluted CALL DNA-unwinding protein. It was found that the serum was capable of detecting such protein at a concentration as low as 3.5 μg/ml.

Treatment of Human Metaphase Chromosomes with Purified Protein. Human metaphase chromosome preparations were made after 72 hr of culture with 2% phytohemagglutinin M and 120 min of colchicine arrest (1 mg/ml), 10 min of 0.075 M hypotonic KCl treatment, and fixation with methanol:acetic acid (3:1, v/v) (12). The slides were kept at 0° before use. Treatment with the purified protein was performed by flooding a defined area of the slide with the protein solution (1 mg/ml) and 10 mM EDTA for 15 min at 35° in a wet chamber. After treatment, the slide was dipped into distilled water at once to rinse the protein, and immediately stained with Giemsa.
reagent (Harleco, Philadelphia, Pa.; Giemsa solution, 2.5 ml; 0.1 M citric acid, 1.5 ml; absolute methanol, 1.5 ml; and Giemsa buffer, 44.5 ml, with pH adjusted to 6.6 with Na₂HPO₄) for 5 min and rinsed in distilled water. Controls using BSA in identical medium were compared on the same slide.

RESULTS

Isolation of a DNA-binding Protein from a UV-irradiated DS Column (Chart 1). The crude homogenate of CLL cells gave numerous protein bands (at least 16 major ones) in 10% SDS-acrylamide gel electrophoretogram. After passing twice through the DS DNA column from which histones, polymerases, endonucleolytic enzymes, and other proteins with an affinity toward native DNA could be removed, 10 discrete bands remained. The flow-through material was further chromatographed on a UV-irradiated DNA column. Most of the protein was seen in the initial wash. Following stepwise elution, a single peak with 280 nm absorbance could be eluted with 1 M NaCl buffer. The fraction with this protein peak, when applied to the SS DNA column, was eluted in the 2 M NaCl fraction, thus indicating that this protein bound to irradiated DS and unirradiated SS DNA. The ³H-labeled protein showed chromatographic similarities to the unlabeled protein, indicating that it was synthesized during the overnight culture. In 10% SDS-acrylamide gel electrophoresis, 2 adjacent discrete protein bands with about 85% purity shown by densitometry tracing were observed, with estimated molecular weights of 13,000 and 11,000, unlike the reported E. coli DNA-unwinding protein and gene 32 protein (Chart 2). The K₅₀ of the protein without SDS and mercaptoethanol treatment on a column of Sephadex G-75 gel corresponded to K₅₀ of a reference protein of molecular weight of 25,700 (chymotrypsinogen).

With special care taken, about 5 mg of the purified protein could be obtained from 10¹¹ CLL cells through 2 runs on a DS DNA column and 2 runs on UV-irradiated DS DNA column. The stepwise improvement of purity could not be readily assessed with our isolation techniques because of the constant presence of DNA nucleases until the end step was reached. Three attempts at isolating this protein were made from normal lymphocytes previously purified by Ficoll:Hypaque density gradients (3). Proteins that had the characteristic of causing DNA helix-coil transition were isolated in very small amounts (somewhat less than 20 µg from 10¹⁴ lymphocytes). Whether they were identical to the CLL protein was further studied by the immunocmchemical method (see below).

The Nucleolytic Activities. By using the method of Center et al., we found that this protein isolated from a DNA-cellulose column containing UV-irradiated DNA did not degrade the circular DNA of φx-174 RFI in any way detectable by nitrocellulose filter technique or by alkaline sucrose gradient sedimentation. When SS [³H]DNA was used as substrate to detect predominantly exonuclease activity, we did not observe any loss of the acid-precipitable radioactivity. Because of the fact that RNase has been found to cause destabilization of the DNA double helix, RNase assay was performed by spectrophotometric method and acid-precipitation technique. While RNase A
caused immediate degradation of the RNA, no detectable changes were observed with the CLL protein at identical protein concentration.

The Effect of Protein on DNA Helix-Coil Transition (Chart 3). We had determined that the prevention of hypochromic shift of SS DNA induced by the addition of Mg\(^{2+}\) requires a protein:DNA weight ratio of somewhere between 6:1 and 10:1 (see “Materials and Methods”). Experiments of this nature were then performed routinely at such weight proportions. Initially crude extracts, flow-throughs from the UV-irradiated DNA column, and all fractions eluted at different salt concentrations were tested. Only the 1 M eluate was found to prevent the Mg\(^{2+}\)-induced hypochromic shift without any detectable nuclease activity. This protein was also found to cause a gradual hypochromicity change of poly[d(A-T)] eventually reaching the level consistent with 100% denaturation (e.g., by heat) at 35° over a 15-min period. The \(T_m\) of poly[d(A-T)] in the presence of this protein was lowered by 20° in SSC 1:10. However, its reassociation was significantly delayed. Because our effort was to see whether the CLL protein possessed more specific effects on altered DNA, a similar experiment was performed on UV-irradiated DNA. We subsequently found that the protein also enhanced such transition in UV-irradiated calf thymus DNA that has previously been extensively sheared. In this case, the enhancement was less marked than in poly[d(A-T)], probably because of its higher \(T_m\). At a constant temperature of 35°, only a 1.17-fold increase in \(A_{260}\) was observed. However, a reduction of \(T_m\) of 17° by this protein was seen as the temperature further rose.

The Effect of Protein on Incision of UV DNA by UV-specific Endonuclease. The UV-specific endonuclease from \(M.\) \textit{luteus} makes SS nicks in native UV-irradiated DNA. When DNA of average dimer distance of about 24,000 daltons was used as substrate for this enzyme, a linear relationship was observed between the estimated size of DNA substrate and time of reaction (Chart 4). The reaction was essentially complete in 75 min. With the addition of this protein at a weight ratio with DNA of 10:1 (25 µg of protein and 2.5 µg of DNA), a facilitation of enzymatic incision was demonstrated. The reaction was complete in 30 min. Molecular weight determination by alkaline sucrose gradients confirmed that the 2 separate degradation products of DNA at 75 min and 30 min were identical.

The Effect of Protein on Metaphase Chromosomes. When cells in metaphase are treated with colchicine (1 µg/ml) for 120 min, disruption of spindle protein and contraction of chromosomes, probably as a result of lack of stretching by spindles, can be observed. These metaphase chromosomes are frequently stained with Giemsa or acetoorcein for cytogenetic analysis. When this protein was added to the prestained metaphases, a marked lengthening and segment formation of the chromosomes were seen (Fig. 1). The effect was demonstrable in virtually all metaphases but was not found in those treated with the buffer alone or with BSA in the same buffer. The frequency or pattern of segmentation differed from the G-banding described in human chromosomes (19).

Detection of Protein among Other CLL Patients, a CML Patient, and Normal Subjects. The rabbit IgG antiserum prepared from the purified protein of a CLL patient’s lymphocytes reacted with the extracts of the same patient from crude stage of preparation to purified protein. It also reacted with the extracts from 4 different patients (Chart 5). Thus, it was useful in determining the immunological identity at various stages of purification. When we tested the extracts isolated from normal lymphocytes of comparable protein concentration to the CLL extracts and leukocytes from a patient with CML, precipitin lines were not observed. This antiserum was normally capable of detecting this protein at 3.5 µg/ml concentration. The observation suggests that the normal extracts either

Chart 4. Left, profiles of elution from a Sepharose 4B column (0.5 x 10 cm) of 'H-labeled UV-irradiated DNA before and after treatment with UV-specific endonuclease (uvendo) from \(M.\) \textit{luteus}. After treatment, the DNA is denatured before application to the column. Right, kinetics of the reaction of uvendo on irradiated DNA with and without the CLL protein at 37°. \(1/D\) is the reciprocal of the elution volume and is a measure of DNA strand length [modified from the work of Carrier and Setlow (4)].
DNA-unwinding Protein in Human Leukemia

Chart 5. Ouchterlony double diffusion to show reactivity between rabbit antisera and proteins purified after a DS DNA cellulose and a UV-irradiated DS DNA-cellulose column from cells of 4 CLL patients, 1 CML patient, and a normal donor. The protein concentration of all 6 specimens was in the range of 150 μg/ml. Clockwise from top well: CLL (W. Y.), CLL (P. E.), CML, CLL (B. H.), normal and CLL (H. S.). No precipitin lines were noted between serum (center well) and CML, and between serum and normal. The heavy lines converged between CML's indicating same identity. They were IgG, whereas lighter lines were IgM by immunoelectrophoresis.

Fig. 1. Two sets of representative human chromosomes belonging to groups A, C, and D are compared under the same magnification (top, protein treated; bottom, BSA control). The metaphase chromosomes were prepared according to the method of Hungerford (12). The slide was divided into 2 parts, 1 of which was flooded with BSA and the other with the UV-specific protein, each in 0.05 M NaCl buffer, at 35° for 15 min. Note the size difference and the change in staining characteristics (segmentation).

have a small amount of this protein below the level of detection or they do not contain the identical protein.

**DISCUSSION**

Isolation of gene 32 product from T4-infected E. coli by Alberts and his coworkers has provided a useful model for the studies of such proteins in other systems. Our effort in this respect has also been greatly aided by Alberts and Herrick. The isolation of CLL proteins has had technical difficulties less frequently encountered in the bacterial or phage system. The nonspecific proteins are present in larger quantities (about 10^-10 g/cell), and numerous nucleases are annoying contaminants. Our study used a simple and repetitiously useful method for isolating DNA-binding proteins in a complex cell system. It is the earliest report of isolation of a DNA-unwinding protein from a human neoplasia. It also provides information for future investigations of various DNA-binding proteins in mammalian or human cells.

The interesting effects of this DNA-binding protein from cells of 4 CLL patients on DNA are its enhancement of helix-coil transition and facilitation of the activity of UV-specific endonuclease from M. luteus. Since CLL cells are more active in excisional DNA repair, compared with normal lymphocytes, its presence may offer an explanation for such biological differences between CLL and the normal condition. Furthermore, the enhanced rate of this repair-type incision induced by the protein in a cell-free system appears to be in better agreement with that observed in UV-irradiated intact cells in that the initial DNA breakdown after the insult, presumably due to endonuclease incision, is completed within the 1st 30 min after UV (A. T. Huang, unpublished data). To determine whether such protein is a requirement in excisional or recombinational DNA repair will require further studies with the use of systems that offer more accurate quantification. Our preliminary effort in isolating proteins of similar nature from normal lymphocytes indicates that a similar protein may be present, but in much smaller amounts per cell, and that it does not react to the rabbit antiserum prepared from CLL protein. Thus, it appears that there may exist a qualitative difference between normal lymphocytes and these leukemic cells.

It is our contention that the CLL protein possesses both DNA-unwinding and UV endonuclease facilitation properties. A recent report (21) that a functional gene 32 product is apparently required for UV repair in T4 in vivo
supports our view that local denaturation of DNA may facilitate the enzyme-substrate association for excisional or recombinational DNA repair.

The effects this protein exerts on metaphase chromosomes are chiefly lengthening of the chromatids and segmentation, as seen in Chart 5. It has been reported that fixed chromosomes still possess the property to elongate or contract, dependent upon the environment (18). We suspect that the CLL DNA-unwinding protein may have caused uncoiling of the tertiary structure of certain segments of the DNA, resulting in segmentation and elongation. By using this protein, we hope to study the chromosomes under electron microscopy in order to delineate the mechanism involved and to study the structure of the complex mammalian chromosomes.

We think that the presence of this unusual protein specifically in CLL cells may be related to their leukemic character. The question arises as to whether this or related proteins are to be found in other leukemias and, if so, with what quantitative and qualitative variations. It is imperative to look first for such proteins in a variety of leukemias, as well as to ascertain that they are obligatory concomitants of CLL.

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