Association of a Possible DNA Ligase Deficiency with T-Cell Acute Leukemia¹

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ABSTRACT

DNA ligase activities were determined in blood samples of 431 different cases of lymphoblastic and nonlymphoblastic leukemia. Less activity was observed in samples from lymphoblastic patients. RNA translation together with ligase immunoprecipitation experiments show that in T-cell acute lymphoblastic leukemic cells, no ligase is translated. This deficiency is correlated with the presence of more breaks in the DNA from these kinds of leukemia which results in altered DNA. This DNA can be ligated by the addition of exogenous ligase. This is the first demonstration of a ligase deficiency in leukemic human cells. These results are discussed in terms of chromosome abnormalities and rearrangements of genes coding for enzymes involved in DNA replication and repair.

INTRODUCTION

The most important target for carcinogenicity appears to be DNA rather than RNA (1). One critical event which leads to mutagenicity seems to be semiconservative DNA synthesis on a damaged template (2). DNA synthesis (either replication or repair) requires several enzymes such as polymerases (3), DNA glycosylases, endonucleases (4), and other related enzymes (5). Among DNA metabolizing enzymes, DNA ligases are of special interest. Enzymes capable of joining DNA fragments were discovered in prokaryotic cells more than 20 years ago (6, 7). Following their identification to DNA ligase in 1967 (8, 9), their physiological role was assessed by the inability of mutants defective in the enzyme to repair their DNA (10). In eukaryotes, the stimulation of ligase activity during rat liver regeneration (11, 12) or lymphocyte division (13) suggests important function(s) of this enzyme in the proliferation processes.

For these reasons, DNA ligase activities were studied in different kinds of normal human immunocompetent and leukemic cells (14). Based upon 431 cases of different kinds of leukemias, the present results show a low or undetected ligase activity in T-ALL.4 We also present data showing that the DNA displays more breaks in these cells.

MATERIALS AND METHODS

Preparation of Cells

Normal lymphocytes were obtained from the Centre Regional de Transfusion Sanguine de Rennes to whom the authors are indebted. WBC were separated from RBC on Ficoll gradients and washed three times for 15 min at 1500 rpm in a Jouan refrigerated centrifuge at 4°C.

Leukemic cells were obtained either from the Hematology Laboratories, CHR de Brest, Rennes Pontchaillou, and Fontenoy or Hôtel Dieu de Paris, France. Classifications were performed by the Laboratoire de Cytologie CHR Rennes or Hôtel Dieu de Paris to whom the authors are also indebted.

DNA Ligase Assays: Preparation of Extracts

To test for enzyme activity, 20 × 10⁶ WBC were homogenized in 0.5 ml of extraction buffer, 20 mM Tris (pH 7.4), 0.5 M KCl, 2 mM dithiothreitol, 0.2% Nonidet P-40, and 0.8 mM phenylmethanesulfonyl fluoride (Sigma). The extracts were sonicated for three 15-s intervals (dial setting 1; Alcatel Sonicator, Paris, France) followed by centrifugation at 140,000 × g for 60 min. Cell extracts (250 µl) were layered on a top of sucrose gradient, 5–20% in 0.5 M NaCl-1 M EDTA-2 mM dithiothreitol-50 mM Tris-HCl, pH 7.4, and centrifuged for 15 h at 45,000 rpm in a SW55 rotor. Fractions of 240 µl were collected and tested for enzyme activity.

The ligase assay was determined after separation on sucrose gradient using a modification (13) of the method described by Modrich and Lehman (15) which measures the resistance of ligated [³²P]poly[da-T₈₅] to exonuclease III. The ligase assay was performed as follows. The incubation mixture contained, in a total volume of 100 µl, 45 mM Tris-HCl (pH 7.8, 4 mM MgCl₂, 0.3 mM ATP, 1 µM [³²P]poly[dA-T₈₅] (corresponding to 115 × 10⁶ cpm), 20 µg bovine serum albumin, 5 mM dithiothreitol, and 0.40 µl enzyme solution. After 30 min of incubation at 37°C, the mixtures were incubated for 3 min in a boiling water bath. Ten units of exonuclease III (New England Nuclear) were added and the assays were incubated at 37°C for 45 min.

The samples were precipitated with trichloroacetic acid (5% final concentration), filtered through Whatman GFC filters, and counted. One unit of DNA ligase activity is defined as the amount converting 1 nmol of poly(dA-T₈₅) to the exonuclease III-resistant form in 30 min under the assay conditions. The activities were routinely checked using a second assay (13) modified from the method of Olivera (16). A third assay was also occasionally used. This assay is based upon ligation of pBR322 and further analysis by agarose electrophoresis (17).

Proteins were determined according to the method of Bradford (18).

Extraction and Translation of RNA

Normal human thymus was obtained from Thoracic Surgery, Pontchaillou Hospital, Rennes, France. Total RNA from 1 g thymus or 2 × 10⁶ WBC from T-ALL or ANLL was extracted according to the method of Chirgwin et al. (19) as modified by Raymondjean et al. (20). Thymus or blast cells were homogenized with a Polytron during three periods of 15 s at maximum speed in 10 volumes of 5 mM guanidine thiocyanate-1 mM EDTA, pH 7.4-100 mM sodium acetate, pH 5.5-Sarkosyl (2%)-β-mercaptoethanol, 5%. After 30 min of 10,000 rpm centrifugation at 20°C, the supernatant was layered on 5.7 M CsCl containing 0.1 mM EDTA, pH 7.4, and centrifuged at 155,000 × g for 16 h at 20°C. The pellet was redissolved in 1 ml of a buffer containing 10 mM Tris-HCl, pH 7.4-5 mM EDTA-1% Sarkosyl. RNA was precipitated in 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.6), 70% ethanol, and absolute ethanol and finally stored at −80°C in water. The integrity of DNA was checked.

The in vitro translation in the rabbit reticulocyte system was as described previously (21).

Microinjection into axolotl eggs was as described previously (22). A glass micropipet was used to inject 10 nl of fluid containing RNA into the egg near the animal-vegetal axis. No leakage was observed. After determination of the RNA quantity required for optimal results, 10 ng were injected. Pools of injected eggs were maintained in Steinberg solution (23) for 9 h at 18°C. Controls received vehicle in 10 nl. The enzyme was extracted as described above.

Immunoprecipitation of Human DNA Ligase

IgG from polyclonal antibodies were purified by gel filtration on a Sephadex G-200 column (23). Prior to specific immunoprecipitation,
0.5 × 10^6 cpm of trichloroacetic acid precipitable material were incubated with normal rabbit serum and a suspension of Staphylococcus aureus to reduce nonspecific binding. The sample was specifically precipitated with anti-ligase IgG.

DNA Analysis

Nucleoid Sedimentation Technique. Blast cells (10^6) in 50 μl of phosphate buffered saline were added to 500 μl of lysis solution (2 M NaCl-10 mM EDTA, 50 μg/ml ethidium bromide-10 mM Tris-HCl, pH 8.0-0.5% (v/v) Triton X-100). This solution was overlaid on 4.6 ml of a 15–30% sucrose gradient containing 2 M NaCl, 1 mM EDTA, 50 μg/ml ethidium bromide, and 10 mM Tris-HCl, pH 8.0. The gradients were kept in the dark for 30 min at 20°C and centrifuged for 1 h at 20,000 rpm at 20°C in a Beckman SW55 rotor. The positions of nucleoid bands were visualized with an UV lamp at 254 nm (24).

Alkaline Elution Assay for Fresh Human Cells. The alkaline elution method was essentially the modification (25) of the method described by Kohn and Ewig (26). Normal lymphocytes (5 × 10^6) and leukemia blast cells from ANLL and T-ALL patients were loaded separately onto 2-μm-pore size polyvinyl chloride filters (Millipore Corp.). They were lysed for 1 h with a solution containing 1.5% sodium dodecyl sulfate-0.02 M EDTA (pH 10.0) containing proteinase K (0.5 mg/ml). The filters were washed with 0.02 M EDTA and eluted with 0.02 M EDTA adjusted to pH 12.3 with tetraphenylammonium hydroxide. Eluted and retained DNAs were measured by enhancement of diamino acid dihydrochloride fluorescence (26).

Alkaline Sucrose Gradient Sedimentation of DNA from ALL and ANLL Blasts. To minimize shear, DNA was prepared by lysing cells suspended in an agarose insert, following a modification of the procedure of Van Der Ploeg et al. (27). Blasts were rapidly centrifuged and resuspended in 0.9% NaCl-20 mM EDTA-phosphate buffered saline, pH 7.4, at a concentration of 10^6 cells/ml, diluted with an equal volume of 1% low gelling agarose at 37°C (Sigma). The suspension was rapidly transferred to 40-μl sample holders and allowed to cool on ice for 2 min. Solidified blocks of agarose termed inserts were submitted to a lysis solution containing 5% sodium lauroylsarcosinate, 0.5 M EDTA (pH 9.5), and 2 mg proteinase K/ml. The assay mixtures were incubated at 50°C overnight. After incubation, the lysis solution was removed and the inserts were washed 10 times with 10 mM Tris-HCl (pH 8.0)-1 mM EDTA. Each agarose block was transferred to 10 mM Tris-HCl (pH 7.6)-5 mM MgCl₂-5 mM ATP either with or without 1 unit of T₇ DNA ligase (Boehringer) as indicated. The reaction was stopped by adding 0.9 unit of alkaline phosphatase (Sigma) for 45 min at 37°C. After two extractions with 500 μl of 1% 2-mercaptoethanol, 30 μM [³²P]ATP (New England Nuclear; 40 Ci/mmol), and 15 units/ml of T₇ infected Escherichia coli 5'-polynucleotide kinase (New England Nuclear) at 37°C until a plateau of incorporation was reached (usually 30 min). The nonincorporated radioactivity was eliminated by washing the inserts on GFC filters with 1% low gelling agarose (Sigma) melted at 65°C and aliquots of 50 μl were layered in 300 μl of lysis solution containing 5% sodium dodecyl sulfate-0.02 M EDTA (pH 10.0) containing proteinase K (0.5 mg/ml). The filters were washed with 0.02 M EDTA and eluted with 0.02 M EDTA adjusted to pH 12.3 with tetraphenylammonium hydroxide. Eluted and retained DNAs were measured by enhancement of diamino acid dihydrochloride fluorescence (26).

RESULTS

DNA Ligase Activity in the Cells from Different Types and Subtypes of Leukemia. DNA ligase activity in leukemia has never been studied on large populations. Results for the enzyme activity in the peripheral WBC of 431 patients are shown in Fig. 1. In general, a lower activity was observed in lymphoblastic than in nonlymphoblastic leukemia and it was not detected or absent in T-ALL (Fig. 1A). In ANLL or chronic myelogenous leukemia a consistently higher activity was found with a maximum in the M₂ subtype of ANLL (Fig. 1B). Ligase activity measured in normal human lymphocytes was found to be 1.4 ± 0.4 (SD) units/10⁶ cells (N = 15 individual donors).

Similar determinations on nonmalignant cell lines and T-cell chronic lymphocytic leukemia showed activities close to that of normal lymphocytes.

The possibility of an artificial origin for this observation, e.g., the presence of a nuclease in T-ALL blast cells, was tested by exposing pBR322 DNA to various cell extracts. As shown in Fig. 2 no significant nuclease activity was observed in assays of T-ALL cells (Fig. 2, lane b) while some nuclease activity was observed in ANLL extracts (Fig. 2, lane c).

Another problem could be the sensitivity of the tests used to measure the ligase activity. T-ALL and ANLL cell extracts were tested regarding the ligation of EcoRI digested pBR322 (17). As shown in Fig. 3 this assay demonstrates the lack of DNA ligase activity in T-ALL cells. Still another possibility exists that ligase activity is inhibited in T-ALL cell extracts. However, the addition of active ligase representing equal amounts of protein from ANLL to sedimentation fractions from T-ALL did not show any inhibition of the enzyme.

Eventually, such an observation on a specific DNA metabolizing enzyme raises the question of whether related others are also affected. DNA polymerases α and β were tested. Activities for T-ALL were, respectively, 0.8 ± 0.27 (SEM) and 0.4 ± 0.18 nmol dNTP incorporated/60 min/10⁶ cells (N = 21). They were 0.95 ± 0.30 and 0.51 ± 0.12 (N = 37) for ANLL. The high SEM indicated a wide range of activities but significant levels have always been observed. Values in normal lymphocytes were, respectively, 0.65 ± 0.15 and 0.30 ± 0.10 nmol dNTP incorporated/60 min/10⁶ cells (N = 15).

Mechanisms of the Observed Absence of Ligase in T-ALL Cells. RNA was extracted from normal human thymocytes, T-ALL, and ANLL blasts and used for in vitro translation testing. The results are presented in Fig. 4. It is clear that when RNAs...
DNA LIGASE DEFICIENCY/LEUKEMIA/DNA BREAKS

Fig. 2. Effect of ALL and acute myelogenous leukemia extracts on exogenous DNA after testing different protein concentrations ranging from 0.01 to 1.2 mg/ml. Five μl (6 μg of protein) of ANLL or ALL extracts were added to 400 ng of pBR322, incubated for 2 h at 37°C, and run on a 1% agarose gel at 100 V for 1 h. Lane a, 100 ng pBR322 in the absence of extracts; Lane b, pBR322 plus ALL extracts; Lane c, pBR322 plus ANLL extracts. Then DNA was visualized in a 2-μg/ml solution of ethidium bromide under UV. Migration was from top to bottom.

Fig. 3. Ligation of cohesive ends of pBR322 by enzymes in ANLL and T-ALL cell extracts. DNA ligase was assayed at 37°C in propylene tubes in a mixture (20 μl) of 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 10 mM dithiothreitol, 0.1 mM ATP, 0.20 μg of digested pBR322 DNA, and 5 μl of enzyme solution containing 0.5 mg/ml of protein. Lane a, digested plasmid (control); Lane b, Tₐ DNA ligase; Lane c, extracts from ANLL; Lane d, extracts from T-ALL cells. DNA was analyzed as indicated in the legend to Fig. 2.

are incubated with a reticulocyte lysate, DNA ligase activity is found only with RNA from thymocytes and ANLL blast cells but not with T-ALL RNA. When the mRNA from T-ALL was translated into reticulocyte lysate apparently normal protein synthesis occurred, thereby showing that this RNA had the capacity for protein translation (Fig. 4B).

However, other translation systems like the amphibian egg are known to be more efficient than the reticulocyte lysate (28, 29). RNA was injected into axolotl eggs according to techniques currently used in the laboratory (21). As depicted in Fig. 5, in each case, the endogenous (axolotl) peak of activity was observed (fraction 3) while no activity was found in the absence of RNA or with the one extracted from T-ALL cells. mRNA was extracted from a second series of injected eggs. As shown in Fig. 5B, the efficiency of the translation of the mRNA from T-ALL cells was comparable to that from other sources. Furthermore, other proteins from T-ALL are translated in the axolotl egg. This is the case for human polymerases α and β.⁶

The evolving hypothesis is that the protein may be present in an inactive form. This hypothesis was checked using antibodies specific for human DNA ligase. Fig. 6 shows the result of immunoprecipitation of the translation products of RNA from T-ALL and ANLL. No protein was immunoprecipitated in the translation product of RNA from T-ALL (Fig. 6, Lane a) while the protein was found in the ANLL extracts (Fig. 6, Lane b).

Studies at the DNA Level. Since the most obvious conse-

⁶ Unpublished results.
The sequence of DNA ligase deficiency is the presence of DNA breaks, this finding prompted us to study the DNA in these cells. With the use of the nucleoid migration technique (24), DNAs from fresh cells from ALL and ANLL patients were compared. As shown in Fig. 7, nucleoid sedimentation in ANLL was very significantly faster than in ALL. This experiment was repeated for 53 samples, and an important difference (Fig. 7A) in the relative sedimentation rates of nucleoids was observed.

To further ascertain the observation that DNA in ALL is of a structure different than the one extracted from ANLL blast cells, DNA was studied using the alkaline elution assay. When DNAs from T-ALL cells, ANLL cells, and normal lymphocytes were compared, a significantly greater elution rate was found for T-ALL (Fig. 8) and this observation is indicative of more breaks in this kind of cell (30).

To confirm the observation that DNA in ALL has properties different from that extracted from ANLL cells the two kinds of DNA were centrifuged on alkaline sucrose gradients. The $^{32}$P labeling was performed after insertion and lysis of the cells into agarose blocks. This method does not result in significant chromosomal DNA breakage (27) and, using HindIII digested pBR322 inserted in agarose, we are able to demonstrate that DNA remains accessible to the ligation process (Fig. 9, inset). As shown in Fig. 9 (dashed lines), an apparent difference was observed in the molecular weight of $^{32}$P labeled DNA extracted from ALL or ANLL cells. However, when the embedded DNA was submitted to exogenous T4 DNA ligase, DNA obtained from ALL migrated as a higher molecular weight structure (Fig. 9A, solid line). For no apparent reason more DNA was recovered. No modification was observed for the ANLL DNA in the presence of ligase (Fig. 9B, solid line).

One possible cause may be that the time course of the ligation process in relation to the 5'-$^{32}$P labeling can result in an
artifactual modification of DNA. To check this point the insert analysis technique was repeated with the samples already studied for nucleoids. Regardless of whether the ligation was performed before or after 5'-32P labeling, no change in the sedimentation was observed with ANLL cells whereas the peak of radioactivity was changed from fraction 12 to fraction 6 with ligated DNA from ALL cells. On the basis of sedimentation of DNA extracted individually from 24 T-ALL and 29 ANLL cases we found that the migration of 32P labeled DNA from the former is different.

In order to assess the nature of 32P labeled DNA, experiments with [3H]thymidine incorporation followed by 5'-32P incorporation were performed. For this purpose, blast cells were submitted to phytohemagglutinin stimulation. The coincidence of the 3H and 5'-32P radioactivity indicated that the [5'-32P]DNA population was a major one. Finally, the possibility that residual DNA-adenylate complexes (30) from DNA ligase may cause the migration difference observed after action of DNA ligase on DNA from ALL was tested by incubation in the presence of [2'-3H]ATP. No 3H radioactivity was present in the gradient in extracts incubated under these conditions.

DISCUSSION

Based upon the determination of ligase activity using three different technical approaches, it can be concluded that, as compared to other kinds of leukemia, the activity is low or undetected in the 77 cases of T-ALL studied in an overall leukemic population of 431.

To our knowledge, this is the first time that a DNA ligase deficiency is reported in human T-ALL. We believe that this observation is of great significance since acute leukemia affects up to 3/100,000 people (32) although low activity is also observed in non-T and non-B Burkitt's lymphoma.

A ligase deficiency has been found recently in Bloom's syndrome cells (33, 34). In these studies, the deficiency was limited to a form I ligase isolated by fast protein liquid chromatography-Sepharose or AcA34 chromatography. In the present report, no ligase II has been found by these two methods for leukemic cells; this observation is in agreement with other results obtained in hepatocytes (12) or on human leukemic cells (14). Unlike Bloom's syndrome ALLs, leukemic cells are of specifically restricted clonal origin (35-37). We report that the deficiency appears to be restricted not only to the T-lineage but also to malignant immature T-cells.

Of importance also is the observation that this deficiency does not affect other closely related DNA replication and repair enzymes. Although a great variability was observed DNA polymerase α and β activities were found in the different types of leukemic cells. This is in agreement with the results of Hutton and Bolli (38).

Another important question is about the molecular basis of such a ligase deficiency. RNA was extracted from T-ALL and ANLL cells and translated in reticulocytes. No ligase activity was observed although this system can translate the ligase mRNA (21). To confirm this point the RNA was injected into the axolotl egg, which is a very efficient translation system (22). No translation of the ligase mRNA was found indicating that no messenger can translate for ligase. However, other proteins like polymerases do translate, showing that the RNA is active.

Still another possibility exists that the protein can be translated in an inactive form. For this reason translation products were immunoprecipitated but no ligase was found. This raises the possibility of gene inactivation associated to chromosomal translocation. Eventually, the gene encoding for DNA ligase either may not be transcribed and/or translated or may be altered. These observations require further investigation of the molecular mechanisms leading to the leukemias and are of crucial importance for understanding the observed phenomenon.

We then studied the DNA in T-ALL using different ap-
proaches. Nucleoid sedimentation showed that the relative migration rate of nucleoids from T-ALL was lower than the one from ANLL. Ethidium bromide dose responses were performed (results not shown) and they show that there are indeed more breaks in T-ALL than in ANLL DNA (24).

However, because of the difficulty in comparing nucleoid from different origins such an observation was confirmed by the alkaline elution technique (25). These findings were also further established by the migration of 32P labeled DNA from T-ALL on alkaline sucrose gradient. From this observation, it can be concluded that the 5'-32P labeled DNA from ALL has a structure different than the one from ANLL blast cells. Moreover, this ALL DNA is accessible to ligation and its apparent molecular weight on alkaline gradients is considerably increased. These results are consistent with the occurrence of either more numerous or more readily ligtatable sites in the DNA of ALL blast cells. We believe these results indicate a selective structural modification of DNA from ALL cells as opposed to other cell types examined.

The hypothesis that an abnormal chromosomal pattern is intimately associated with the malignant phenotype is already ancient (39). However, the most relevant observation was the identification of the Philadelphia chromosome in chronic myelogenous leukemia (40), and more recent searches (41–43) for chromosomal abnormalities in leukemia have resulted in the finding that a large majority of patients with acute leukemia have an abnormal karyotype (44–46). This number may even reach 100% in some laboratories (46).

It was observed that chromosomes carrying genes related to nucleic acid biosynthesis are frequently involved in rearrangement associated with hematological malignancies (47). Since DNA ligase is closely involved in DNA replication and repair biosynthesis it was of interest to study the DNA ligase level in different types of leukemia. Ligase activity was found to be absent or poorly efficient in T-ALL. This defect may account for the longer DNA synthesis time required in these cells when compared to normal T-cells (48).

Of special importance is the fact that the DNA extracted from T-ALL blasts can be resealed by T4 DNA ligase. This method allowed the detection of DNA strand breaks after in vitro treatment with chemical carcinogens. Mutât. Res., 130: 283–294, 1984.


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