Elevated NAD(P) Glycohydrolase Activity: A Possible Enzymatic Marker for Malignancy in Burkitt’s Lymphoma Cells

By H. Skala, G. M. Lenoir, A. L. Pichard, M. Vuillaume, and J. C. Dreyfus

A comparative analysis of enzymatic activities has been performed on 47 human continuous lymphoid lines: 22 tumors derived from Burkitt’s lymphoma lines, 6 other lymphomatous long-term cultures, and 19 nonmalignant EBV-transformed lymphoblastoid cell lines. On the 5 activities determined on the cell extracts, 4 showed no significant differences between the various lines. They included adenosine diphosphoribose incorporation, glucose-6-phosphatase dehydrogenase, cyclic-AMP phosphodiesterase, and glutathione reductase. However, striking differences of activity were found for the enzyme, NAD(P) glycohydrolase (EC 3.2.2.8). Activity levels were, as a mean, four times higher in Burkitt’s lymphoma-derived cell lines than in nonmalignant control lines, and the difference was highly significant (p < 0.02). All Burkitt cell lines containing translocations of chromosome 8 with either chromosomes 2, 14 or 22 showed an increased activity. The specificity and significance of this possible enzymatic marker of Burkitt’s lymphoma cells is discussed.

The finding of biochemical markers of malignant cells has been one of the aims of cancer research in recent years. Up to now, no enzyme has been found to be reliable as a specific marker for cancer in tumors or leukemias.1 Much work has been devoted to phosphorylation and protein kinases in the study of normal and abnormal cell growth. More recently, studies on adenosine diphosphate (ADP) and polyADP ribosylation showed that these modifications of proteins may play an important role in cell transformation.2 ADP ribose transferase activity has been described as moderately increased in lymphoma cells;3 polyADP ribose level is increased in carcinogen-treated cells,4 and its synthesis diminishes when granulocytes5 or intestinal epithelial cells6 differentiate. No clear relationship, however, could be established between the dividing rate or other properties of malignant cells and the production or polymerization of ADP ribose.

Human long-term lymphoid cell lines appeared to be suitable for such an exploration. Two main types of permanent human lymphoid lines are available: the first are represented by those established from malignant clones (mainly lymphomas or leukemias), and the majority are Burkitt’s lymphoma (BL) derived lines. The second group consists of nonmalignant B cells, which had acquired the capacity of growing continuously in culture following “immortalization” by Epstein-Barr virus (EBV). The latter are designated as human lymphoblastoid cell lines (LCL).7 Whereas all LCL are polyclonal in origin and necessarily contain the EBV genome, BL lines may or may not contain the viral DNA and usually have cytogenetic changes characteristic of the malignant clone from which they are derived: most of these lines display chromosomal translocation between chromosome 8 and either chromosome 2, 14, or 22.8

In this investigation, we have measured total incorporation of ADP ribose into proteins from 47 different human lymphoid cell lines. We also determined the activity of the enzyme, NAD(P) glycohydrolase, since this enzyme is a possible producer of ADP ribose from its precursor NAD+. In addition, the activity of two enzymes as controls, e.g., glucose-6-phosphate dehydrogenase and cyclic-AMP phosphodiesterase, was measured. Finally, we also determined the activity of glutathione reductase in a number of cell lines; this enzyme is coded by a gene located on chromosome 8.9

The major result of our investigation is a striking difference in NAD(P) glycohydrolase activity between malignant BL lines and LCL, a difference that might be linked with their specific translocation involving chromosome 810 and then may be related to their malignant state.

Materials and Methods

Cell Lines

The characteristics of the lymphoid lines used in this study are summarized in Table 1. The 22 Burkitt-type lymphoma lines were established from patients originating from both endemic and nonendemic regions; 16 of them do contain the EBV genome. Twenty of these lines possess a specific BL translocation: 14 with t(8;14), 4 with t(2;8), and 2 with t(8;22), whereas BJAB and its EBV genome counterpart, BJAB-B95, lack such translocation.

The 19 lymphoblastoid cell lines were all established in our laboratory, by G.M.L. from peripheral lymphocytes of individuals with or without disease as mentioned in Table 1. In two cases, IARC 99 and IARC 100, the LCL were established from the normal peripheral blood lymphocytes from two BL patients whose lymphoma lines (IARC BL16 and IARC BL18, respectively) have also been included in the studies.
Six non-BL lines of malignant origin and lacking the EBV genome have also been studied. They were derived from leukemia (2), myeloma (1), and histiocytic (1) or lymphocytic (2) lymphomas.

The cells were cultivated in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 100 IU/ml of penicillin, and 100 μg/ml streptomycin. The cells were collected 2 or 3 days after medium change and were still in exponential growth phase. The cell suspensions were washed twice in phosphate-buffered saline and stored at −70°C as frozen cell pellets until enzyme analysis. Cells frozen at −70°C were extracted in a phosphate buffer, pH 6.4, EDTA 10^{-4} M containing 0.5% Triton X-100, then frozen and thawed 3 times. The homogenate was centrifuged 30 min at 30,000 g.

**Reagents**

Nicotinamide 1N\textsuperscript{6} ethenoadenine nucleotide (phosphate) (ε-NAD and ε-NADP) were purchased from Sigma Chemical Co., St. Louis, Mo. and NAD labeled with \textsuperscript{3}P from New England Nuclear, Boston, Mass. All other chemicals were of the highest available purity and came from Merck (Darmstadt, FRG) and Sigma.

**Enzymatic Methods**

For the determination of NAD(P) hydrolase activity, we employed a method using a fluorescent analogue of NAD(P): the Nicotinamide 1N\textsuperscript{6} ethenoadenine dinucleotide (phosphate) (ε-NAD or ε-NADP). The assay was basically that described by Barrio et al.\textsuperscript{11} The intensity of fluorescence (excitation 300 nm, emission 410 nm) of ε-NAD(P) increased about tenfold upon hydrolysis, corresponding to the separation of the nicotinamide and the ethenoadenine moiety:

ε-NAD(P) \rightarrow ε-ADP ribose + nicotinamide

The reaction mixture contained in a total volume of 200 μl of 50 mM phosphate buffer (pH 6.4), 10^{-4} M EDTA, and the substrate ε-NAD or ε-NADP 2 \times 10^{-4} M. At this concentration we are at saturation of the substrate. Incubation was performed at 37°C. We adapted a monitor to the fluorimeter (Aminco-Bowman) so that the rate of enhancement of fluorescence could be followed. The slope was linear during the first minutes of the reaction and was proportional to the concentration of proteins. NAD glycohydrolase activity was expressed as a relative enhancement of fluorescence/minute/milligram protein.

In some experiments in the presence of nicotinamide, which quenches fluorescence, activity of NAD hydrolase was assayed by the classical cyanide method of Kaplan.\textsuperscript{12} The enzyme was assayed in 0.1 M phosphate buffer, pH 7.2, using 6 \times 10^{-4} M NAD as substrate.

The ADP ribosyl transfer assay mixture contained 50 μl of a supernatant of 30,000 g of cells extracted in a phosphate buffer, pH 6.4, containing EDTA 10^{-4} M, Triton X-100 0.5%, and NAD (10 μCi) 2 \times 10^{-3} M. Incubation was performed for 10 min at 30°C, then 10-μl aliquots of the incubation mixtures were spotted onto a Whatman 3 mm filter paper (8 x 9 cm) previously treated with a 2% (w/v) trichloroacetic acid (TCA) solution according to Marie et al.\textsuperscript{13} The paper was then stirred in a 10% (w/v) TCA solution at 4°C. Noncovalently bound radioactivity was eliminated by electrophoresis in methanol 5%–acetic acid 7.5%. After electrophoresis, the paper was rinsed with water, alcohol, acetone, and then dried. The spotted samples were counted for radioactivity in a LKB counter.

Glucose-6-phosphate dehydrogenase and glutathion reductase activities were determined according to Beutler.\textsuperscript{14} Cyclic-AMP phosphodiesterase was determined according to Richard and Cheung.\textsuperscript{15}

**RESULTS**

ε-NAD(P) activities in the different cell lines are represented in Table 1. In our results, we have pooled the activities ε-NAD and ε-NADP because first, the

<table>
<thead>
<tr>
<th>BL Lymphoma Lines</th>
<th>EBV</th>
<th>Cytogenetic</th>
<th>No. of Independent Determinations</th>
<th>Mean</th>
<th>Standard Deviation</th>
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<tr>
<td>Ramos</td>
<td>−</td>
<td>8;14</td>
<td>7</td>
<td>11.2</td>
<td>3.9</td>
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<tr>
<td>Ramos/B95</td>
<td>+</td>
<td>8;14</td>
<td>3</td>
<td>8.6</td>
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<tr>
<td>J BL 2</td>
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<td>2;8</td>
<td>4</td>
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<tr>
<td>IARC/BL 7</td>
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<td>2;8</td>
<td>4</td>
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<tr>
<td>B J A B / B95</td>
<td>+</td>
<td>2;8</td>
<td>3</td>
<td>7.6</td>
<td>3</td>
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<tr>
<td>RA JI</td>
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<td>8;14</td>
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<td>2.34</td>
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<td>DG 76</td>
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<td>8</td>
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<td>P3HRI</td>
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<tr>
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<tr>
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<td></td>
<td></td>
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<td>1.2</td>
<td>0.76</td>
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</table>

*EBV-positive BL cells obtained by in vitro infection of EBV-negative BL clones.
activities were quite similar, and second, evidence was presented that both activities (NAD and NADP hydrolases) are associated with a single enzyme.

The means of activities for the other enzymes tested were the following: phosphodiesterase, 1.42 ± 1.09 nM/mg/min; glucose-6-phosphate dehydrogenase, 0.045 ± 0.02 U/l/mg; glutathion reductase, 0.046 ± 0.016 U/l/mg; and ADP ribose incorporation, 1172 ± 570 cpn/mg. No significant differences between the different lines could be noted for these four determinations.

Taking the value of NAD(P) glycohydrolase (2.85) as a borderline, all BL lines (except one) had values over 2.85, whereas all lymphoblastoid lines (except one) were under 2.85. The non-Burkitt-derived lymphoma lines were equally distributed around this value (Fig. 1).

DISCUSSION

In an attempt to identify enzymatic characteristics of malignant cells we studied 47 lymphoid cell lines available in the laboratory of one of us. The center of our investigation was the incorporation of ADP ribose from labeled NAD by extracts from the various cell lines. No consistent pattern emerged from our results. NAD(P) glycohydrolase, which is one of the enzymes leading to the formation of ADP ribose, was also assayed in order to compare its activity to that of transfer of ADP ribose to TCA-precipitable material. Striking differences were found, with values varying for more than one order of magnitude. Taken as a group, the mean activity of the Burkitt’s lymphoma cell lines was four times higher than that of the non-Burkitt lines, and the difference highly significant. Except for one line in each group, there was no overlapping between the two groups. These results can be discussed along several lines.

Validity of NAD(P)ase Determinations

The determination of NAD(P) hydrolase activity has been made by a fluorimetric technique, which is much faster and more sensitive than NAD(P) measurement. However, it is not entirely specific (as is also true of the classical technique), since any cleavage of the dinucleotide would enhance fluorescence. The major potential interfering enzyme would be pyrophosphatase. In order to eliminate this interference, we performed our assays at pH 6.4. The optimum pH of action of pyrophosphatase is alkaline and only at pHs...
above 7.5 was significant interference by other NAD hydrolyzing enzymes detected.\textsuperscript{17}

Moreover, when the assays were performed in the presence of 0.1 \textit{M} nicotinamide, we observed an inhibition of the reaction. Nicotinamide is known to be an inhibitor of NAD(P) glycohydrolase, while it has no action on pyrophosphatase. These two arguments support our conclusion that the activity we observe is really due to the enzyme NAD(P) hydrolase.

In addition, the intervention of a nuclear NADase was made unlikely by the constant ratio that was found when NAD or NADP was taken as a substrate. The nucleus contains some NADase but no NADPase activity according.\textsuperscript{18}

\textbf{Specificity of NAD(P)ase as a Marker}

In addition to NAD(P)ase and to ADP ribose incorporation, three enzymes were assayed in a number of cell lines. No significant variations were observed for glucose-6-phosphate dehydrogenase or cyclic-AMP phosphodiesterase. Glutathione reductase was included in this study because it is coded by a gene assigned to chromosome 8, and no significant differences were observed.

\textit{Significance of the Increase of NAD(P) Glycohydrolase in Burkitt’s Cell Lines}

The cell lines that showed an increase in NAD(P) glycohydrolase activity have two features in common: one or origin, which is Burkitt’s lymphoma, and the other cytogenetic, the rearrangement of chromosome 8. Three types of translocations of chromosome 8 were observed in our material: 8-14 (the most frequent in our lines), 8-2, and 8-22. All types showed an increase, but the level of activity does not seem to be related to
the type of specific translocation observed. The lowest enzymatic activity was observed in the only BL line that does not possess this specific translocation, suggesting that the elevated activity may be related to this specific chromosomal change observed in BL cells, in particular to the involvement of chromosome 8 zone, 8q24. However, the role of chromosome 8 is difficult to assess; the fact that glutathione reductase is not influenced is of little value, since the gene for this enzyme is on the short arm of the chromosome and the translocation always on the long arm. The chromosomal location of NAD(P) glycohydrolase is not known. The only attempt we could make in that respect was a determination of this enzymatic activity in the red cell membranes of a child with a trisomic anomaly for chromosome 8. No difference with the controls could be found.

Because our lines showed no gross chromosomal abnormalities, while the range for NAD(P) glycohydrolase activity was more than 1–10, no simple gene dosage effect can be explanatory, and regulatory effects must be postulated. These effects could be of two kinds. (1) Chromosome 8 could be involved. If the enzyme gene was on that part of the chromosome that is translocated, it could be in a state of activation greater than in its normal location. (2) If chromosome 8 is not specifically involved in the phenomenon, general regulatory effects would be the cause. This could be ascribed either to the Burkitt-derived cell in general or to indirect effects of chromosome rearrangements. Such regulatory modifications could be expected despite the fact that they have been observed only rarely.

The presence or the absence of the viral genome in BL cells does not seem to be related to enzymatic activity. However, we must note that the activity of BL BJAB was greatly enhanced following in vitro conversion by EBV. This was not observed in the case of the Ramos line, the original activity of which was extremely high, nor CHE with intermediate values.

In order to exclude that elevated activity was a genetic characteristic of the individuals with BL tumors, we have tested two pairs of lymphoma/LCL derived from the same individuals. In both cases, BL cells were found with enzymatic values over 2.85, whereas lymphoblastoid lines had less than 2.85 values. This was also confirmed when testing other LCL from cancerous patients.

**Consequences of NAD(P) Glycohydrolase Increase in Activity**

One may wonder whether the above results could bring to Burkitt’s cells a selective advantage or disadvantage. In fact, very little is known about the role of the enzyme. Experiments have decisively shown that the enzyme is located in the external part of plasma membranes. Its mode of action is not known, and our results showed that a preliminary hydrolysis of NAD is not a prerequisite for ADP ribosylation. We can conclude that ADP ribose radioactivity incorporated into proteins is due only to the activity of ADP ribose transferase. This is supported by the fact that added cold ADP ribose does not decrease incorporation (unpublished). Therefore, the level of glycohydrolase does not interfere with ADP ribose incorporation. Pending the discovery of its physiologic activity, no conclusion can be drawn regarding the importance of glycohydrolase activity. It has even been claimed that in blacks, in red cell membranes at least, the enzyme is deficient, but this does not cause any apparent damage.21

Two recent studies may help in furthering the understanding of the possible role of this enzymatic difference. It would be worthwhile testing whether the monoclonal antibody prepared by Wiels et al. recognizes molecules related to NAD(P) glycohydrolase activity. Furthermore, we have to find out whether the high NAD glycohydrolase found in BL cells is related to the malic enzyme deficiency observed in most malignant lymphoid cells by Povey et al.23 In conclusion, it appears that a significantly high activity of NAD(P) glycohydrolase activity is a biochemical marker of Burkitt’s lymphoma-derived lymphoid lines. It cannot be stated whether this increase is characteristic of Burkitt’s lymphoma or of rearrangements of chromosome 8. The physiologic significance and importance of this finding cannot yet be ascertained. It seems to us, however, that this constitutes the first positive enzymatic marker that can be confidently ascribed to a definite class of malignant cells.

**REFERENCES**

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