Primary Thrombocytethmia: Clonal Origin of Platelets, Erythrocytes, and Granulocytes in a Gd^B/Gd^Mediterranean Subject

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A patient with primary thrombocytethmia, who was heterozygous for glucose-6-phosphate dehydrogenase deficiency (Gd^B/Gd^Mediterranean), was investigated to test for the clonal origin of this myeloproliferative disorder. In order to assess somatic cell mosaicism in various tissues, we have made use of the different rate of utilization of 2-deoxyglucose-6-phosphate, an analog of glucose-6-phosphate, by normal glucose-6-phosphate dehydrogenase and by the Mediterranean variant: the results demonstrate that essential thrombocytethmia is a clonal disease involving the erythrocytic, granulocytic, and megakaryocytic series, without affecting monocytes, T lymphocytes, and non-T lymphocytes.

THE X-LINKED isoenzyme system of glucose-6-phosphate dehydrogenase (G6PD) has been used to demonstrate, in affected heterozygous women, the clonal origin of several myeloproliferative disorders, including chronic myeloid leukemia, primary polycythemia, and myelofibrosis. Since in each female cell only one X chromosome is genetically active, cell populations from heterozygotes will express enzyme mosaicism if derived from many cells, whereas a clonal origin will be indicated by the expression of one type of enzyme only. This approach has been confined to women heterozygous for the A and A' variants of G6PD, which are easily distinguishable from the normal B enzyme by electrophoresis. We have recently shown that the different utilization of 2-deoxyglucose-6-phosphate (2dG6P), an analog of the normal substrate glucose-6-phosphate (G6P), by the normal enzyme and the Mediterranean variant of G6PD allows a convenient and accurate determination of mosaicism in cell populations from heterozygous individuals. Since the normal enzyme utilizes 2dG6P less than 4% as rapidly as the normal substrate and the Mediterranean variant around 30%, the utilization by the heterozygote is related to the degree of mosaicism. By this method we can detect a population of normal or G6PD-deficient cells as small as 5%. It is then possible to extend studies on the clonal origin of many neoplastic diseases to women heterozygous for the Mediterranean variant of G6PD.

We describe here the results obtained in a woman with primary thrombocytethmia and heterozygous for Mediterranean G6PD, whose different hemopoietic cell populations were isolated and tested for clonal origin with the 2dG6P method. Essential thrombocytethmia appears to be a clonal disease involving not only megakaryocytes, but also the erythrocytic and the granulocytic cell lines.

CASE REPORT

A 73-yr-old woman of Sardinian origin was admitted in November 1980 for thrombocytosis. Two years before mild hypothyroidism had been diagnosed; moderate anemia was present, and a bone marrow examination showed an increased number of megakaryocytes. The platelet level at that time was normal.

On admission to our department, physical examination was negative; no significant bleeding episodes were reported; the patient was slightly anemic (Hb 9.6 g/dl with normal mean corpuscular volume), white cells count was 10,000/cu mm with a normal differential; the platelet count was around 800,000/cu mm. Giant platelets and platelet aggregates were seen in the peripheral blood. Platelet aggregation in vitro was below normal range. Serum iron, B12, and folate levels were within normal limits; there was still mild hypothyroidism. The leucocyte alkaline phosphatase score was 20 (normal values 15–100). X-rays of the chest, gastrointestinal tract and i.v. pyelography were normal. A bone marrow aspirate revealed a great increase in immature megakaryocytes showing nuclear and cytoplasmic abnormalities. Chromosomal analysis from bone marrow cells showed a normal karyotype. Follow-up of the patient for several months showed a steady increase of platelet count (up to 1,200,000/cu mm) and she was started on periodical thrombocytapheresis.

No other modifications of the previous hematologic findings occurred during that time.

At the time of diagnosis, the patient’s erythrocytes were assayed for G6PD, and the activity was 2.2 U/100 ml RBC (normal value 140 ± 20 U). The patient’s two sons were then tested and found to have normal G6PD values. The possibility of a clonal modification involving erythrocytes and other cell series in a G6PD heterozygote with thrombocytethmia then arose, and further studies were undertaken to verify this hypothesis.

MATERIALS AND METHODS

Blood samples and skin biopsies were obtained from the patient and, as controls, from a G6PD Mediterranean deficient man, a G6PD Mediterranean heterozygote, and a normal subject; at least three tests were carried out on different occasions on the same subject.
**PRIMARY THROMBOCYTHEMIA**

**Erythrocytes**

Red blood cells were separated from white cells and platelets by repeated passages of whole blood through α-cellulose/microcrystaline cellulose (Sigma Chemical Company, St. Louis, Mo.).

Red cell lysates were prepared and G6PD activity measured. The hemolysates were further purified by the single step of affinity chromatography on 2',5'-ADP Sepharose 4B (Pharmacia, Uppsala, Sweden). No 6-phosphogluconic dehydrogenase activity was present. The enzyme was then characterized according to the WHO methods. 2dG6P relative utilization was determined during the characterization procedure of the purified enzyme. Purified enzyme preparations were tested rather than hemolysates because of the low G6PD activity present in Mediterranean red cells. Samples from normal and heterozygote controls were purified in the same way to obtain comparable results.

**Lymphocytes**

Defibrinated blood from the patient and from three controls was layered on a Ficoll-Hypaque gradient. The cells at the interface were collected and incubated at a concentration of 2 x 10⁹/ml in RPMI 1640 medium plus 20% FCS in a Petri dish at 37°C for 1 hr. Nonadherent cells were collected and rosetted with neuraminidase-treated sheep erythrocytes. Rosetting cells were counted, and the lymphocytes were spun through a second Ficoll-Hypaque gradient. Rosettes were collected from the bottom of the gradient and non-T lymphocytes at the interface. Sheep erythrocytes were lysed by osmotic shock. The purity of the cell preparation was assessed on cytocentrifuged slides stained with May-Grunwald-Giemsa (MGG) and α-naphthyl acetate esterase (ANAE). Contamination by monocytes was less than 5% in all preparations. The cells were then sonicated for 60 sec in a lysing solution containing Triton 1%, NADP⁺, and DFP. The cell lysates were centrifuged at 16,000 g for 20 min, and the supernatants tested for G6PD activity and 2dG6P relative utilization.

**Monocytes**

Mononuclear cells were isolated from defibrinated blood through a Ficoll-Hypaque gradient. Cells were washed and resuspended at the bottom of a three-phase Percoll gradient; the density of the lower solution was 1,232 g/ml, of the intermediate 1,061.5 g/ml, and the top 1,049 g/ml. The gradient was centrifuged at 400 g for 5 min, and monocytes were collected at the interface between 1.061.5 and 1.049. Contamination by lymphocytes was less than 3% by examination of cytocentrifuged slides stained with MGG and ANAE. Monocytes were then lysed and treated as for lymphocytes.

**Granulocytes**

Defibrinated blood was spun through a Ficoll-Hypaque gradient; the erythrocyte-rich fraction was sedimented with dextran and granulocytes were isolated from the supernatant. Cell preparation purity was confirmed, and the cells were treated as above.

Platelet contamination was checked in all white cell preparations with direct count and found to be negligible. Erythrocyte contamination was eliminated with osmotic shock.

**Platelets**

Platelets were isolated by the method of Baenziger and Majerus. Briefly, platelets were separated from whole EDTA-anticoagulated blood with successive low-speed centrifugations. The final pellet was subjected to osmotic lysis to eliminate red cell contamination. White cells were absent from the final preparation. Platelets were then disrupted by sonication and the lysate assayed for G6PD.

**Hair Follicles, Skin, and Fibroblasts**

Scalp hair roots were collected from different parts of the occipital region; an average sample consisted of 20–25 hair roots with visible bulbs and sheaths, which were lysed for 15 min in a solution containing Triton 1%, NADP⁺, and DFP. The lysate was centrifuged and assayed for G6PD activity and 2dG6P relative utilization. Skin biopsies were obtained from the patient and the three controls. Part of the skin biopsy was directly tested; fibroblasts were cultured, harvested, and assayed as described for hair roots.

**Enzyme Assay**

G6PD activity of all supernatants of the crude lysates and of the purified G6PD from red cells was measured from the rate at which NADPH was formed under two conditions: in the presence of the normal substrate (G6P) and in the presence of the analog (2dG6P) at the same concentrations (0.6 mM). The results with 2dG6P were expressed as percentage of the enzyme activity with G6P. This percentage is called relative 2dG6P utilization. Enzyme assays were performed at 25°C on a Beckman 25 K Spectrophotometer (Beckman Instruments, Palo Alto, Calif.) with a recording system. All experiments were carried out in duplicate.

**RESULTS**

Relative 2dG6P utilization of the different cell populations isolated from the patient and three healthy controls (a normal subject, a woman heterozygote for G6PD Mediterranean, and a G6PD-deficient man) are reported in Table 1. The numbers listed represent

<p>| Table 1. Relative 2dG6P Utilization Values of Different Cell Populations From the Patient and the Controls (%) |
|--------------------------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Patient</th>
<th>Normal</th>
<th>Heterozygote</th>
<th>G6PD-Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin biopsy</td>
<td>10.7</td>
<td>3.4</td>
<td>10.2</td>
<td>29.4</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>11.7</td>
<td>3.4</td>
<td>10.9</td>
<td>31.2</td>
</tr>
<tr>
<td>Hair follicles</td>
<td>11.3</td>
<td>4.2</td>
<td>11.3</td>
<td>33.2</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>30.8</td>
<td>4.5</td>
<td>8.9</td>
<td>29.9</td>
</tr>
<tr>
<td>Platelets</td>
<td>31.3</td>
<td>4.2</td>
<td>9.6</td>
<td>31.4</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>32.0</td>
<td>3.7</td>
<td>9.2</td>
<td>30.6</td>
</tr>
<tr>
<td>Monocytes</td>
<td>12.3</td>
<td>3.5</td>
<td>9.7</td>
<td>30.1</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>12.7</td>
<td>3.8</td>
<td>9.7</td>
<td>29.6</td>
</tr>
<tr>
<td>Non-T lymphocytes</td>
<td>15.1</td>
<td>3.4</td>
<td>9.8</td>
<td>30.9</td>
</tr>
</tbody>
</table>
the mean of the results obtained in at least three experiments performed at different times. The coefficient of variation ranged between 4.8% and 9.6%.

2dG6P relative utilization is very uniform in all the various cell populations isolated from each of the three control subjects, being less than 4% in the normal subject, about 10% in the heterozygote, and about 30% in the G6PD-deficient man. 2dG6P values of the patient show two different patterns: monocytes, T lymphocytes, and non-T lymphocytes have a 2dG6P utilization similar to that of tissues of nonhemopoietic origin (hair follicles and skin fibroblasts) and consistent with a heterozygous condition. Eleven clones derived from cultured skin fibroblasts were also tested; relative 2dG6P utilization was around 30% for six of them and within the normal range for the other clones, thus confirming the presence of both G6PD isoenzymes in nonhemopoietic tissues. Platelets, granulocytes, and erythrocytes exhibited a relative 2dG6P utilization consistent with a fully G6PD-deficient phenotype, thus supporting a clonal origin from a common precursor cell (Fig. 1).

**DISCUSSION**

We have recently demonstrated the possibility of evaluating the degree of mosaicism in mononuclear cells from heterozygotes for the Mediterranean G6PD variant on the basis of the different 2dG6P utilization by the normal and the mutant enzyme. This extends the investigation on the clonal origin of various neoplastic diseases to these heterozygote subjects. We have used this approach for the first time to investigate the clonal origin of different cell populations from a patient with essential thrombocythemia and heterozygous for Mediterranean G6PD. The first assay for G6PD activity on the patient’s erythrocytes gave values compatible with a fully deficient phenotype; the possibility of homozygosity for the Mediterranean gene was ruled out by family examination. Extreme X inactivation was excluded by examination of skin biopsy and cultured fibroblasts, which expressed a 10% relative 2dG6P utilization, corresponding to a 60/40 G6PD*/G6PD_ mosaicism. Hair follicles were also tested and the results were identical to those obtained from cultured fibroblasts.

We then examined purified cell populations isolated from the patient’s blood and the results listed in Table 1 clearly indicate that monocytes, T and non-T lymphocytes share the same 2dG6P relative utilization of tissues of nonhemopoietic origin; these cell lines are therefore not involved in the malignant process. On the other hand, erythrocytes, platelets, and granulocytes showed a 2dG6P utilization compatible with a clonal origin from a common precursor cell where inactivation of the normal G6PD gene bearing X chromosome had occurred, with consequent expression of Mediterranean G6PD only.

Figure 1 shows that for healthy controls, 2dG6P utilization ratio between different cell populations and nonhemopoietic tissues is always constant, around 1, consistent with the usual finding that the degree of G6PD mosaicism is the same in all tissues of an individual. In the patient, the ratio is normal when 2dG6P utilization of tissues of nonhemopoietic origin is compared with that of monocytes, T and non-T lymphocytes, while the expression in the case of erythrocytes, granulocytes and platelets clearly indicates clonal proliferation. Essential thrombocythemia is then a clonal disease involving a multipotent stem cell common to red cells, granulocytes, and platelets.

The same has been proved true for chronic myeloid leukemia, acute myelogenous leukemia, and primary polycythemia, but in some of these studies macrophages from bone marrow cultures have been reported to share clonal origin with granulocytes and plate-
Our findings are in apparent contrast with the undoubted expression of an homogenous heterozygote cell population.

We conclude that primary thrombocythemia is a clonal myeloproliferative disorder that involves a multipotent stem cell common to the granulocytic, erythrocytic, and megakaryocytic lines. Moreover, the 2dG6P method has been proved a reliable technique to investigate clonal origin of neoplastic diseases in G6PD Mediterranean heterozygote subjects.

ACKNOWLEDGMENT

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