Prognostic Value of the Cysteine Proteases Cathespins B and Cathepsin L in Human Breast Cancer

Christoph Thomssen, Manfred Schmitt, Lothar Goretzki, Peter Oppelt, Lothar Pache, Peer Dettmar, Fritz Jänicke, and Henner Graeff

Frauenklinik [C. T., M. R. C.], Institut für Medizinische Statistik und Epidemiologie [L. P.], and Institut für Allgemeine Pathologie und Pathologische Anatomie [P. D.], der Technischen Universität München, Klinikum rechts der Isar, Ismaninger Strasse 22, 81675 Munich, Germany.

ABSTRACT

The lysosomal cysteine proteases cathespins B and cathepsin L have been implicated in tumor spread and metastasis. To evaluate the prognostic impact of these proteases for disease-free survival and overall survival in breast cancer, the antigen content of cathespins B and cathepsin L was determined using ELISA in tumor cytosol fractions of 167 breast cancer patients and in cytosols of 29 benign breast tissue specimens.

Median values of 856 ng versus 76 ng cathespins B/mg protein and of 428 ng versus 56 ng cathepsin L/mg protein were found in tumor versus benign cytosol fractions. A positive correlation between cathespins B and cathepsin L (r = 0.32, P = 0.0000, Spearman test) was found. Cathespins L was inversely correlated to hormone receptor status (P = 0.0014, Mann-Whitney U test) and to the presence of tumor necrosis (P = 0.009, Mann-Whitney U test). There were no correlations of cathepsins B or cathepsin L to tumor size, axillary lymph node status, age, menopausal status, tumor grading, and vessel invasion. To perform univariate analyses of disease-free survival, optimal cutoff points were determined by isotonic regression and classification and regression trees analysis. Patients with a high content of cathespin B (>1092 ng/mg protein) or cathepsin L (>376 ng/mg protein) in their primary tumors had a statistically significantly higher risk of recurrence than patients with a low content of cathespins B or cathepsin L (5-year disease-free survival: cathespin B, 70% versus 52%, P = 0.04; cathepsin L, 83% versus 52%, P = 0.0002). Median follow-up was 39 (range, 6–73) months. Multivariate analysis for disease-free survival showed that cathepsin L is a strong and independent prognostic factor with a prognostic impact comparable to that of axillary lymph node status and grading. We conclude that both cathespins B and cathepsin L may serve as prognostic factors for tumor recurrence in human breast cancer. These data underline the significance of tumor-associated proteolysis for invasion and metastasis.

INTRODUCTION

Invasion and metastasis in solid tumors require the action of tumor-associated proteases. Proteolytic enzymes such as plasmin, uPA, cathepsins B, D, and L, and members of the metalloprotease family are involved in the degradation of components of the extracellular matrix and the basement membranes. The extent of proteolysis is limited by specific inhibitors, e.g., PAI-1/2, steves, cystatins, and tissue inhibitors of metalloproteinases (1–6). In this process the lysosomal cysteine proteases cathespins B and L are instrumental by directly degrading extracellular matrix constituents and converting inactive pro-uPA into enzymatically active uPA (7–9). uPA and its inhibitor PAI-I seem to play a key role in the activation and/or regulation of tumor-associated proteolysis (10, 11). Furthermore, clinical studies in breast cancer patients have demonstrated that the antigen content of uPA and PAI-I in breast cancer tissue extracts may serve as remarkable prognostic factors since they are strongly correlated with DFS and overall survival (12–14). In the present study, we analyzed whether the tissue content of the lysosomal cysteine proteases cathespins B and cathepsin L is also of prognostic value in breast cancer.

MATERIALS AND METHODS

Patients. Between February 1987 and November 1991, 167 breast cancer patients undergoing primary surgical treatment at the Department of Obstetrics and Gynecology, the Technische Universität München, were enrolled into this study. Those patients who had metastatic disease at the time of diagnosis of their primary tumor were not included. Surgical treatment was performed either by modified radical mastectomy or by breast-conserving surgery, including axillary lymph node dissection. Premenopausal patients with lymph node involvement (n = 31) received adjuvant chemotherapy consisting of six courses of cyclophosphamide (600 mg/m² i.v., day 1), methotrexate (40 mg/m² i.v., day 1), and 5-fluorouracil (600 mg/m² i.v., day 1) repeated every 21 days. Postmenopausal patients received adjuvant hormone therapy (tamoxifen; n = 56). No adjuvant therapy was administered to 68 of the node-negative patients, 7 node-negative patients received tamoxifen, and 4 node-negative patients received chemotherapy. The patients were followed up by clinical check-ups every 3 months for a median time of 39 (range, 6–73) months. Follow-up was defined as the interval between date of primary surgery and date of last observation or death. Patient characteristics are listed in

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2 To whom requests for reprints should be addressed, at Frauenklinik und Poliklinik der Technischen Universität München, Ismaninger Strasse 22, 81675 Munich, Germany.
3 The abbreviations used are: uPA, urokinase-type plasminogen activator; PAI-1/2, plasminogen activator inhibitor type 1/type 2; CI, confidence interval; DFS, disease-free survival; RR, relative risk.
Table 1. They correspond to a typical cohort of breast cancer patients. The control group consisted of 29 patients with benign breast tissue.

Tissue Extraction. Breast cancer tissue specimens were obtained at primary surgery, selected by the pathologist, and stored in liquid nitrogen until extraction. Tissue specimens without indication of necrosis were obtained from the central part of the tumor; the cooling cycle was not interrupted. Tissue specimens from patients with benign breast disease or of the normal mammary gland served as controls (normal breast, mastopathy, and fibroadenoma). Deep-frozen specimens of 200–500 mg were pulverized using the Micro-Dismembrator (Braun-Melsungen, Melsungen, Germany) set to 30 s at maximum power. The resulting still frozen powder was immediately sus-

Observation time (mo)
Median (range) 39.0 (6.0–73.0)
Age (yr) Median (range) 55.6 (27.3–82.2)
Menopausal status
Pre-/perimenopausal 64 38.3%
Postmenopausal 103 61.7%
Tumor size (cm)
median (range) 2.5 (0.5–11.0)
≤2.0 55 32.9%
>2.0, ≤5.0 cm 95 56.9%
>5.0 17 10.2%

Histopathology
Invasive ductal 147 88.0%
Invasive lobular 13 7.8%
Medullary 5 3.0%
Tubular 2 1.2%

No. of axillary lymph nodes involved
0 79 47.3%
1–3 46 27.5%
4–10 24 14.4%
>10 18 10.8%

Hormone receptor status
Positive 126 75.4%
Negative 41 24.6%

Grading a
1 5 3.0%
2 97 58.1%
3 65 38.9%

Vessel invasion
Absent 134 80.2%
Present 33 19.8%

Tumor necrosis
Absent 117 70.1%
Present 42 25.1%
Unknown 8 4.8%

a Grading according to Scarff-Bloom-Richardson.

Cathepsins B and L, Steroid Hormone Receptor, and Protein Analyses. Contents of cathepsins B and L antigen were determined using commercially available ELISA kits (BioASS, Diessen, Germany). Polyclonal rabbit antibodies used in these kits were raised against human spleen cathepsin B and cathepsin L, respectively, and were purified by means of immunoaffinity chromatography with immobilized synthetic cathepsin B or cathepsin L peptides. Specificity of the antibodies was guaranteed by the manufacturer and confirmed by Western blot analyses. These antibodies (capture antibody) were attached by adsorption to the cavities of 96-well polystrene microtiter plates. Breast cancer cytosol fractions (1:40) or cytosol fractions of benign breast tissue (1:10) were subsequently added, and the plates were incubated for 16 h at 4°C. Purified cathepsin B and cathepsin L from human spleen served as the respective standards. The same antibody that was used as the capture antibody was conjugated with horseradish peroxidase and then used as detection antibody. After incubation with 3,3′,5,5′-tetramethylbenzidine, the resulting absorption was visualized at 450 nm and analyzed by a computer-assisted ELISA-microtiterplate reader (ICN-Flow Laboratories, Meckenheim, Germany). According to the manufacturer, intraassay precision of cathepsin B and cathepsin L ELISA was calculated assessing 10 wells of the same concentration of cathepsins B and L, respectively, per plate. For cathepsin B in the range of the standard curve, the intraassay coefficient of variation was between 3.9 and 8.2% and for cathepsin L between 4.0 and 8.5%. Likewise, the interassay precision for 10 determinations was between 3.2 and 10.2% for cathepsin B and between 3.5 and 10% for cathepsin L, respectively. To show the linearity of the tests, breast cancer cytosol fractions with known content of cathepsin B and cathepsin L, respectively, were serially diluted by us and tested; the recovery was greater than 95% of the original concentrations for both cathepsin B and cathepsin L at a dilution range of 1:10–1:100. Protein content was determined using the BCA Protein Assay Reagent kit manufactured by Pierce (Rockford, IL). BSA served as protein standard. Cathepsin B and cathepsin L antigen concentrations were calculated per mg protein. Hormone receptor determinations were performed using the dextran-coated charcoal technique. Specimens were considered to be estrogen or progesterone receptor positive if the hormone receptor content exceeded 20 fmol/mg protein.

Statistical Analyses. To determine the relative prognosis of cathepsins B and in relation to the effect of known prognostic factors in a prospective fashion, DFS was analyzed according to Cox’s proportional hazard model using the BMDP software package (BMDP Statistical Software, Los Angeles, CA) and classification and regression trees analysis as reported previously for uPA and PAI-1 (14). Statistical analyses included continuous as well as discrete covariates, all of which were considered fixed (not time dependent). Determination of the optimal cutoff for cathepsin B and cathepsin L in order to discriminate patients with a low content of cathepsin B or L from those with high values was performed using isotonic regression and classification and regression trees analysis. The value with maximum log rank test was taken for the discrimination of high and low. The 95% CI for this cutoff was calculated according to a test-based method and bootstrap techniques. DFS and overall survival were calculated according to Kaplan-
Meier. The relative risk of relapse and death in regard to high cathepsin B and cathepsin L, respectively, and various established prognostic factors were computed using the Cox model. Correlations were calculated according to Spearman. The relationship of cathepsin B and cathepsin L to clinical and histological prognostic factors was analyzed using the Mann-Whitney U test. All tests were performed at a significance level of $\alpha = 0.05$ (14).

RESULTS

Both cathepsin B and cathepsin L antigen content in primary breast cancer cytosols are considerably elevated in comparison to nonmalignant breast tissue (Fig. 1). For cathepsin B ($n = 167$), a median value of 856 ng/mg protein was determined in breast cancer tissue cytosols as compared to 76 ng/mg protein for benign breast tissue ($n = 29$, $P = 0.0000$). Likewise, for cathepsin L ($n = 167$), a median value of 428 ng/mg protein was determined in breast cancer tissue cytosols in comparison to 56 ng/mg protein for benign breast tissue ($n = 29$, $P = 0.0000$). We found a correlation of $r = 0.32$ between cathepsin B and cathepsin L ($P = 0.0000$, Spearman test). The relationship of cathepsin B and cathepsin L to established prognostic factors was also evaluated. For cathepsin B, no correlations were found to tumor size, axillary lymph node status, age, menopausal status, grading, and vessel invasion. For cathepsin L, we found a correlation with tumor necrosis ($P = 0.009$, Mann-Whitney U test) and an inverse correlation with the hormone receptor status ($P = 0.0014$, Mann-Whitney U test) could be demonstrated. There was no correlation of cathepsin L to tumor size, axillary lymph node status, age, menopausal status, grading, and vessel invasion. To determine the prognostic impact of cathepsin B and cathepsin L in breast cancer in comparison to established risk factors, univariate and multivariate analyses of DFS and overall survival were performed by using Cox’s proportional hazard model. In univariate analysis, the optimal cutoff of cathepsin B for DFS was calculated to be 1092 ng/mg protein. For cathepsin L, a cutoff of 376 ng/mg protein was obtained.

The major new finding is that breast cancer patients with either a high content of cathepsin B ($>1092$ ng/mg protein) or cathepsin L ($>376$ ng/mg protein) in their primary tumors have an increased risk of relapse (Fig. 2). Breast cancer patients with a high content of cathepsin B ($>1092$ ng/mg protein) had a 5-year DFS probability of only 52%, whereas those with low levels showed a 5-year DFS of 70% ($P = 0.0385$). For overall survival, regarding cathepsin B content, no significant difference could be observed.

Cathepsin L is of even higher prognostic impact than cathepsin B. Patients with a high content of cathepsin L ($>376$ ng/mg protein) in their primary tumors had a poor prognosis, too. Similar to cathepsin B, their 5-year DFS probability amounts to only 52%. Patients with low cathepsin L content, however, had a considerably better prognosis: the 5-year DFS probability reaches 83%. This difference is statistically highly significant ($P = 0.0002$). In contrast to cathepsin B, cathepsin L was of prognostic impact for overall survival, too. In this high-risk group, 23 of 92 patients died due to breast cancer whereas
Prognostic Value of Cathepsins B and L in Breast Cancer

Cathepsin B (n=167)  
- cathepsin B < 1092 ng/mg protein
- cathepsin B > 1092 ng/mg protein

Cathepsin L (n=167)  
- cathepsin L < 376 ng/mg protein
- cathepsin L > 376 ng/mg protein

Survival curves for DFS (left) and OS (right) comparing low-risk and high-risk groups for Cathepsin B and Cathepsin L.

Fig. 2 Prognostic significance of cathepsin B and cathepsin L in breast cancer patients in relationship to DFS probability and overall survival (OS) probability (n = 167). Optimized cutoff levels were calculated by isotonic regression and classification and regression trees.

Table 2 Multivariate analysis of cathepsin B and cathepsin L antigen and established prognostic factors in breast cancer

<table>
<thead>
<tr>
<th>Factor</th>
<th>DFS</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td>Axillary lymph node status (node-negative vs. node-positive)</td>
<td>0.0002</td>
<td>0.0000</td>
</tr>
<tr>
<td>Cathepsin L (≤376 vs. 376 ng/mg protein)</td>
<td>0.0002</td>
<td>0.0001</td>
</tr>
<tr>
<td>Grading (SBR ≤2 vs. &gt;2)</td>
<td>0.0018</td>
<td>0.0005</td>
</tr>
<tr>
<td>Vessel invasion (absent vs. present)</td>
<td>0.0004</td>
<td>0.0012</td>
</tr>
<tr>
<td>Hormone receptor status (positive vs. negative)</td>
<td>0.0012</td>
<td>0.0018</td>
</tr>
<tr>
<td>Cathepsin B (≤1092 vs. &gt;1092 ng/mg protein)</td>
<td>0.0385</td>
<td>0.3837</td>
</tr>
<tr>
<td>Menopausal status (post- vs. pre/perimenopausal)</td>
<td>0.0603</td>
<td>0.0628</td>
</tr>
<tr>
<td>Tumor size (≤5 vs. &gt;5 cm)</td>
<td>0.0713</td>
<td>0.0864</td>
</tr>
</tbody>
</table>

* Cox model, P values by log rank test.

only 6 of 74 patients died in the low-risk group (5-year overall survival probability of 69% versus 91%; P = 0.006).

To weigh the relative prognostic strength of cathepsin B and cathepsin L in comparison to other prognostic factors (e.g., axillary lymph node status, grading, tumor size, menopausal status, vessel invasion, and hormone receptor status), multivariate analyses were performed (Table 2). Evidently, the multivariate analyses of DFS and overall survival disclosed that
To our knowledge, our report on breast cancer patients is the first demonstration of the prognostic value of cathepsin B and cathepsin L antigen in patients with solid malignant tumors. A clinical impact of cathepsin B and cathepsin L in breast cancer has already been suggested by Lah et al. (6) and Gabrijelcic et al. (15), applying enzyme activity measurements. Lah et al. (6) also assessed cathepsin B and cathepsin L in breast cancer cytosols, using a different approach. They measured the enzyme activity of cathepsin B and cathepsin L in 45 breast cancer cytosols and using this technique showed a considerable increase in cathepsin B (18-fold) and cathepsin L (53-fold). In our group of 167 breast cancer patients, an approximately 11-fold increase in cathepsin B and an 8-fold increase in cathepsin L antigen content over values determined in benign control tissue extracts were calculated. A direct comparison of antigen content and enzyme activity is not feasible because of obvious differences in methods regarding substrate specificity, specific activity, proenzyme forms, active proteases, and protease-inhibitor complexes (9, 17). Nevertheless, in their relatively small group of 45 patients, Lah et al. (6) observed a relationship of high cathepsin L enzyme activity to prognosis in a univariate manner already after a follow-up of 11–48 months.

In our group of 167 breast cancer patients comprising a typical, representative cohort, cathepsin B and cathepsin L were shown to be of prognostic relevance for DFS after a median observation time of 39 months. Multivariate analysis revealed that cathepsin B was of no independent prognostic value when cathepsin L and established factors were included in the Cox model. This might be due to the positive correlation between cathepsin B and cathepsin L antigen levels. In contrast to cathepsin B, cathepsin L remained a strong and independent prognostic factor for DFS and also overall survival. Evidently, cathepsin L is a strong factor for DFS, in our analysis nearly as strong as axillary lymph node status (Table 2), which is accepted to have a prognostic impact comparable to that of the histomorphological parameters axillary lymph node status and grading. Multivariate analysis for DFS revealed that cathepsin L is one of the strongest prognostic factors with a RR of 4.53 (95% CI, 2.01–9.89; \( P = 0.0001 \)), almost with the same impact as the axillary lymph node status (RR, 4.98; 95% CI, 2.24–11.93; \( P = 0.0000 \)). Cathepsin L is also a strong prognostic factor for overall survival as shown by multivariate analysis (RR, 4.12; 95% CI, 1.53–9.73; \( P = 0.0015 \)) predicting survival probability with a prognostic impact very close in order to that of axillary lymph node status (RR, 4.83; 95% CI, 2.14–10.12; \( P = 0.0000 \)) and grading (RR, 4.41; 95% CI, 1.96–10.01; \( P = 0.0001 \)). In the present multivariate analyses, no independent prognostic value of cathepsin B was found for DFS or for overall survival.

To illustrate the independent prognostic value of cathepsin L, univariate analyses of DFS were performed in node-negative and node-positive patients. In these analyses the cutoff values determined for the entire patient sample were applied (Table 3). Cathepsin L was of significant prognostic value in both the node-negative and node-positive patients. Cathepsin L showed a prognostic value even in node-positive patients receiving adjuvant chemotherapy \( (n = 31, P = 0.03) \) or adjuvant tamoxifen \( (n = 56, P = 0.02) \). For Cathepsin B, a prognostic impact was observed in node-positive patients only.

**DISCUSSION**

Lysosomal cysteine proteases cathepsin B and L have been shown to be associated with tumor invasion and metastasis in solid tumors (4, 15). For more than 10 years, data relating cysteine proteases to tumor progression have been available; mainly based on in vitro investigations or on animal experiments (4, 5, 9). The important new finding in the present study is that tumor levels of cathepsin B and cathepsin L are directly related to the clinical outcome of breast cancer patients. For this purpose, antigen determination of cathepsin B and cathepsin L, as evaluated by ELISA, was applied and shown to be a useful approach to quantify these proteases in tumor cytosol fractions. To our knowledge, our report on breast cancer patients is the first demonstration of the prognostic value of cathepsin B and cathepsin L antigen in patients with solid malignant tumors. A clinical impact of cathepsin B and cathepsin L in breast cancer has already been suggested by Lah et al. (6) and Gabrijelcic et al. (15), applying enzyme activity measurements. Lah et al. (6) also assessed cathepsin B and cathepsin L in breast cancer cytosols, using a different approach. They measured the enzyme activity of cathepsin B and cathepsin L in 45 breast cancer cytosols and using this technique showed a considerable increase in cathepsin B (18-fold) and cathepsin L (53-fold). In our group of 167 breast cancer patients, an approximately 11-fold increase in cathepsin B and an 8-fold increase in cathepsin L antigen content over values determined in benign control tissue extracts were calculated. A direct comparison of antigen content and enzyme activity is not feasible because of obvious differences in methods regarding substrate specificity, specific activity, proenzyme forms, active proteases, and protease-inhibitor complexes (9, 17). Nevertheless, in their relatively small group of 45 patients, Lah et al. (6) observed a relationship of high cathepsin L enzyme activity to prognosis in a univariate manner already after a follow-up of 11–48 months.

In our group of 167 breast cancer patients comprising a typical, representative cohort, cathepsin B and cathepsin L were shown to be of prognostic relevance for DFS after a median observation time of 39 months. Multivariate analysis revealed that cathepsin B was of no independent prognostic value when cathepsin L and established factors were included in the Cox model. This might be due to the positive correlation between cathepsin B and cathepsin L antigen levels. In contrast to cathepsin B, cathepsin L remained a strong and independent prognostic factor for DFS and also overall survival. Evidently, cathepsin L is a strong factor for DFS, in our analysis nearly as strong as axillary lymph node status (Table 2), which is accepted to have the strongest histopathological and clinical factor in breast cancer (18).

The strong prognostic impact of cathepsin L is in line with its assumed function in tumor-associated proteolysis. Cathepsin L exhibits a high capacity to degrade extracellular matrix proteins, e.g., collagen, laminin, and elastin (19). Its proteolytic

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Cathepsin B</th>
<th>Patients</th>
<th>Relapses</th>
<th>DFS probability</th>
<th>( P ) (log rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Node-negative (n = 68)</td>
<td>≤1092 ng/mg</td>
<td>44</td>
<td>6</td>
<td>0.79</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>&gt;1092 ng/mg</td>
<td>24</td>
<td>5</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Node-positive (n = 88)</td>
<td>≤1092 ng/mg</td>
<td>66</td>
<td>17</td>
<td>0.66</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td>&gt;1092 ng/mg</td>
<td>22</td>
<td>12</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Node-negative (n = 68)</td>
<td>≤376 ng/mg</td>
<td>29</td>
<td>1</td>
<td>0.75</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>&gt;376 ng/mg</td>
<td>39</td>
<td>10</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Node-positive (n = 88)</td>
<td>≤376 ng/mg</td>
<td>40</td>
<td>6</td>
<td>0.85</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>&gt;376 ng/mg</td>
<td>48</td>
<td>23</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

* Cutoff values of the entire patient sample were used also in the subgroup analyses. Estimation of DFS probability according to the Kaplan-Meier method, \( P \) values by log rank.

* Node-negative patients with any adjuvant treatment (chemotherapy or tamoxifen) were excluded from the analyses.
power is higher than that of cathepsin B (9, 20, 21). Moreover, cathepsin L is also the strongest known activator of the proenzyme form of uPA (pro-uPA; Ref. 8). There is evidence that degradation of the endothelial basement membranes requires direct tumor cell contact with basement membranes (22). It may well be possible that the action of cathepsin B and cathepsin L in tumor stroma degradation is more important than that of collagenases because of the acidic microenvironment, which is in the range of the pH optimum of cathepsin B and cathepsin L, but not in that of type IV collagenases which require a neutral pH (9). The excreted precursors of cathepsin L have been assumed to be identical to major excreted protein, a protease that is involved in tumorigenesis as known from findings obtained with H-ras-transformed mouse fibroblast cell lines. Joseph et al. (20) described a high correlation of the level of cathepsin L mRNA with the metastatic potential of c-Ha-ras-transformed cell lines. Moreover, Chauhan et al. (19) suggest that cathepsin L is also involved in cell growth regulation by cleaving corresponding control proteins.

As it has already been demonstrated before for uPA, PAI-1, and cathepsin D, tumor-associated proteolytic factors might be suitable candidates for the selection of low- and high-risk breast cancer patients (14). This finding is of considerable clinical interest, especially for the patients with tumor-free axillary lymph nodes, since reliable factors for the estimation of prognosis in this early stage of the disease are urgently needed to individualize adjuvant therapy. Whether or not determination of cathepsin L is able to yield independent prognostic information in node-negative disease in addition to uPA and PAI-1 remains to be elucidated.

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