Pediatric Oncology Group Study of in Vitro Clonal Growth Patterns of Leukemic Cells in Childhood Acute Nonlymphocytic Leukemia as a Predictor of Induction Response

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ABSTRACT

Previous studies have shown that clonal growth patterns of leukemic cells from adult patients with acute nonlymphocytic leukemia (ANLL) have prognostic significance for achieving complete remission (CR). In order to determine if a similar correlation between clonal growth patterns and response to chemotherapy exists in childhood ANLL, bone marrow cells from 189 children with newly diagnosed ANLL were cultured in agar. After 7 days of incubation, colonies (>50 cells), large clusters (20 to 50 cells), and small clusters (4 to 20 cells) were counted. Cultures were analyzed for frequency of clusters and colonies as well as for size of clusters. Two growth patterns significantly associated with poor prognosis for achieving CR were observed: large-cluster growth and high cluster incidence (defined as >400 clusters/10^6 bone marrow cells). The CR rate for the former was 53% (versus 79% for non-Group 1 patients; P = 0.03); the CR rate for the latter was 46% (versus 81% for non-Group 2 patients; P = 0.004). These findings indicate that clonal growth characteristics of leukemic cells from childhood ANLL patients are significantly correlated with response to induction chemotherapy and are useful in identifying a subset of patients with poor prognosis.

INTRODUCTION

The soft-agar clonal assay for hematopoietic progenitor cells has been extensively used in the study of leukemic cell proliferation in adult ANLL. Similar studies in childhood ANLL have not been reported. It has been shown that the clonal growth in soft agar of leukemic cells from adult ANLL patients is typically different from that seen with normal bone marrow cells (1–4). Cultures of normal bone marrow cells in the presence of CSA yield both colonies (>50 cells) and clusters (4 to 50 cells) consisting of granulocytes and monocytes/macrophages. These colonies arise from committed myeloid progenitor cells at different stages of maturation. In contrast, leukemic bone marrow cells from adult ANLL patients generally show abnormal growth patterns as determined by the relative and absolute frequencies of colonies and clusters which develop when the pretreatment cells are cultured. Cultures may yield either no colonies or clusters, or excessive numbers of clusters with reduced or absent colony growth. Moore et al. (2) initially proposed a classification schema for adult ANLL based on these in vitro growth patterns, and a similar system was later proposed by Spitzer et al. (3). These classification schemas were able to identify subsets of adult ANLL patients with a relatively poor prognosis for achieving CR.

In the present study, we have analyzed the clonal growth patterns of bone marrow cells from 189 children with untreated ANLL to determine if in vitro leukemic-cell growth patterns are correlated with remission induction. We have used a modification of the original classification schema of Moore et al. to analyze the observed growth patterns.

MATERIALS AND METHODS

Bone Marrow Cells. After informed consent was obtained, bone marrow aspirations were performed on children with ANLL at the time of diagnosis. Heparinized marrow was added to an equal volume of culture medium and shipped to Emory University via overnight delivery. Median viability of the cells upon arrival was 92% by trypan blue exclusion with a range of from 76 to 99%. The leukemic cells were separated from RBC and mature granulocytes by centrifugation (1000 × g for 15 min) over Ficoll-Hypaque (density, 1.077 g/ml; Sigma). Viability of the cells after Ficoll separation was typically greater than 95%. The light-density fraction contained >80% leukemic cells (as judged by Wright's stained cytocentrifuge preparations). Leukemic cells were classified according to the FAB classification (5). The percentage of patients in each FAB group was as follows: M1/M2 (acute myeloblastic leukemia); M1 and M2 were not separated in the classification; 46%; M3 (acute promyelocytic leukemia); 7%; M4 (acute myelomonocytic leukemia); 33%; M5 (acute monocytic leukemia); 13%; M6 (acute erythroleukemia), 1%.

Hematologically normal bone marrow cells were obtained following informed consent from children with solid tumors without bone marrow involvement. Normal marrow cells were cultured in the clonal assay to assess the growth and differentiation of normal myeloid progenitor cells.

Treatment. Patients were treated according to the Pediatric Oncology Group Phase III ANLL protocol (Protocol 8101). Patients were randomized to receive two induction treatments, with 144 patients receiving Induction Treatment I (DAT) and 45 patients receiving Induction Treatment II (VAD). Induction Treatment I consisted of ara-C (100 mg/m²/day by infusion on Days 1 to 7); daunomycin (either 30 or 45 mg/m²/day on Days 1 to 3, for patients <2 or >2 yr of age, respectively); and 6-thioguanine (100 mg/m²/day on Days 1 to 7). If the bone marrow remained M3 (>25% blasts) or showed progressive disease after 21 days of treatment, the patient was considered to show no response and the study was terminated.

Induction Treatment 2 consisted of ara-C (100 mg/m²/day by infusion on Days 1 to 7); vincristine (2 mg/m²/wk on Days 1, 8, and 15); and dexamethasone (6 mg/m²/day for 21 days). If the bone marrow remained M3 or showed progressive disease after 16 days of treatment, the patient was considered to show no response and the study was terminated.

A patient was considered to have a complete response if an M1 bone marrow (<5% blasts) was achieved with resolution of extramedullary disease and resolution of normal hematopoiesis. Patients with partial responses (M2 marrow with 5 to 25% blasts) were given an additional

Received 1/13/87; accepted 5/7/87.

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This investigation was supported in part by the following grants awarded by the National Cancer Institute, Department of Health, Education, and Welfare: CA-20549; CA-03161; CA-29281; CA-29139; and CA-30969. Also supported by the CURE Foundation of Georgia. Presented in part to the American Association for Cancer Research, Los Angeles, CA (1986).

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2 The abbreviations used are: ANLL, acute nonlymphocytic leukemia; CSA, colony-stimulating activity; CR, complete remission; FAB, French-American-British; DAT, daunomycin-ara-C-6-thioguanine; VAD, vincristine-ara-C-dexamethasone; ara-C, 1-β-D-arabinofuranosylcytosine; GCT-CM, giant cell tumor-conditioned medium.

3 The classification schema proposed by Moore et al. (2) was modified by the authors to include a fifth group (Group 0) of patients who had low MI and low MAC.

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course of treatment and reevaluated. Thirty % of the patients required two courses of treatment to induce complete remission. Patients who failed to complete therapy due to complications (i.e., bleeding) or who developed persistent marrow hypoplasia were not evaluated.

Agar Clonal Assay. The agar clonal assay was a modification of the Robinson-Pike assay (6), as developed by Kubota et al. (7). This assay uses GCT-CM (Gibco) rather than normal peripheral blood cells as a source of CSA. GCT-CM provides a more uniform source of CSA from month to month than do peripheral blood underlayers. In the Kubota study (7), no significant differences in cluster/colony growth were noted among several different types of conditioned media, including GCT-CM and phytohemagglutinin-lymphocyte-conditioned medium. However, it is possible that use of other sources of CSA may affect the clonal growth patterns of some patients.

The Ficoll-separated bone marrow cells were cultured in agar-medium consisting of 0.3% agar (purified; Difco) in Iscove’s modified Dulbecco’s medium (Gibco) with 20% fetal bovine serum, 15% GCT-CM, and 1% penicillin-streptomycin. Culture volumes of 1.0 ml of agar-medium containing 1 x 10^8 bone marrow cells were placed in 35-mm Petri dishes (Falcon) and incubated for 7 days in 7.5% CO2-air at 37°C. Cultures were performed in quadruplicate.

Cultures were scored using an inverted microscope. They were analyzed for the total number of small clusters (4 to 20 cells), large clusters (20 to 50 cells), and colonies (>50 cells).

Morphological Studies. In order to study the morphology of cells in colonies and clusters, cultures were prepared using methylcellulose as a gelling agent. Methylcellulose cultures were identical to agar cultures except that 0.8% methylcellulose was substituted for agar. After 7-days incubation, colonies and clusters were harvested by adding phosphate-buffered saline to the culture dish to dilute the gel and then aspirating the entire culture. The cells were washed 3 times and deposited on a slide using a cytocentrifuge. Cells were then stained with Wright-Giemsa. In some cases, single large colonies were aspirated from the culture with a finely drawn Pasteur pipet and stained individually.

Classification Schema. A modification of the Moore classification schema for in vitro growth patterns was used to classify the bone marrow cultures. Clonal growth patterns were divided into three main categories (types 1, 2, and 3) (Table 1). Type 1 growth contained two subdivisions: (a) no growth (including cultures with persisting single cells but without formation of clones); (b) a low incidence of colonies (<10 colonies/10^6 cultured bone marrow cells) with a normal ratio of clusters to colonies (<15:1), determined from normal bone marrow cultures.

Type 2 growth was characterized by clones containing less than 20 cells (i.e., small clusters without colonies). In type 3 growth, a variable percentage of clusters contained greater than 20 cells, including large clusters and colonies. Type 3 was further divided into three subtypes: (a) cultures containing large clusters of 20 to 50 cells without colonies; (b) both colonies and clusters with an abnormal ratio of clusters to colonies (15:1); (c) both colonies and clusters with a high colony incidence (>10 colonies/10^6 cultured cells). In their studies of adult ANLL, Moore et al. (2) and Spitzer et al. (3) found that patients whose pretreatment leukemia cells showed type 3 growth had a significantly lower probability of achieving CR than did patients with growth of types 1 or 2.

Cultures were also analyzed for total number of clusters (both large and small clusters) regardless of the number of colonies. More than 400 clusters per 10^6 bone marrow cells were considered high cluster incidence, and less than 400 clusters per 10^6 bone marrow cells were considered low cluster incidence. This figure represented the upper limit of cluster growth per 10^6 cells in normal bone marrow cultures.

Statistical Methods. When frequencies were sufficiently large, classical x^2 tests were used to examine differences in CR rates between individual clonal growth patterns (8). Otherwise, an exact test based on the x^2 was used (9). A stepwise multivariate logistic regression analysis was used to determine the significance level of in vitro growth patterns and selected clinical characteristics in the observed outcome (8). The purpose of this analysis was to determine if clonal growth patterns and other clinical characteristics contribute significantly to remission-induction response after adjusting for the remission-induction treatment used (DAT versus VAD). This adjustment was necessary, since in this study induction treatment is known to be significantly correlated with remission-induction rate.

RESULTS

Type 1, consisting of subtypes 1a and 1b, included 54% of the patients studied. Patients with type 1 growth had the highest overall CR rate (82%) (Table 2). Type 1a (no colony or cluster growth) had a CR rate of 81%, and type 1b (colony and cluster growth, with a normal cluster:colony ratio) had a CR rate of 87%. These CR rates were not significantly different from the overall CR rate of 76% (i.e., 144 responders and 45 nonresponders). Cluster counts for type 1b ranged from 2.5 to 83 (median, 16.5), and colony counts from 1 to 10 (all counts are per 10^6 Ficoll-Hypaque-separated bone marrow cells). The cluster:colony ratio for type 1b varied from 2:1 to 15:1.

Type 2 growth (small clusters of <20 cells without colonies) was exhibited by cells from 15% of the patients studied. These patients had a CR rate of 75%. Cluster counts ranged from 3 to 1040 (median, 15).

Type 3 growth, consisting of subtypes 3a, 3b, and 3c, was exhibited by cells from 31% of the patients studied. The overall CR rate for type 3 was 66%. Type 3a (large clusters of up to 50 cells without colonies) contained 10% of the patients and had a CR rate of 53%. Cluster counts in type 3a ranged from 6 to 540 (median, 182). These clusters were noticeably larger (20 to 50 cells) than type 2 clusters, which generally contained 4 to 10 cells.

Type 3b (colony and cluster growth, with a high cluster:colony ratio) contained 10% of the patients and had a CR rate of 68%. Cluster counts ranged from 18 to 2200 (median, 376) and colony counts, from 1 to 9. The cluster:colony ratio varied from 22:1 to 197:1.

Table 2 Correlation between clonal growth pattern of leukemic bone marrow cells and complete remission rate for 189 patients with childhood acute nonlymphocytic leukemia at diagnosis

<table>
<thead>
<tr>
<th>Growth pattern</th>
<th>No. of patients</th>
<th>% of total patients</th>
<th>CR rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a, no growth</td>
<td>72</td>
<td>38</td>
<td>81</td>
</tr>
<tr>
<td>1b, clusters and colonies; normal cluster:colony ratio</td>
<td>30</td>
<td>16</td>
<td>87</td>
</tr>
<tr>
<td>2, small clusters only</td>
<td>28</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>3a, large clusters only</td>
<td>19</td>
<td>10</td>
<td>53*</td>
</tr>
<tr>
<td>3b, clusters and colonies; high cluster:colony ratio</td>
<td>19</td>
<td>10</td>
<td>68</td>
</tr>
<tr>
<td>3c, clusters and colonies; &gt;10 colonies/10^6 BM cells*</td>
<td>21</td>
<td>11</td>
<td>76</td>
</tr>
<tr>
<td>Total</td>
<td>189</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

* Significant (P = 0.03) correlation between large-cluster growth and low CR rate. Other clonal growth patterns were not significantly correlated with CR rate.

* BM cells, bone marrow cells.

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Table 1 Classification schema for clonal growth patterns of leukemic bone marrow cells in pediatric acute nonlymphocytic leukemia at diagnosis, adapted from Moore et al. (2)

<table>
<thead>
<tr>
<th>Type 1</th>
<th>a. no growth (includes cultures with persisting single cells)</th>
<th>72</th>
<th>38</th>
<th>81</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b. colonies (&gt;50 cells) and clusters (&lt;50 cells) with a normal ratio of clusters to colonies (&lt;15:1) and low colony incidence (&lt;10 colonies/10^6 BM cells)*</td>
<td>30</td>
<td>16</td>
<td>87</td>
</tr>
<tr>
<td>Type 2</td>
<td>Small cluster (&lt;20 cells) without colonies</td>
<td>28</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>a. large clusters (20–50 cells) without colonies</td>
<td>19</td>
<td>10</td>
<td>53*</td>
</tr>
<tr>
<td></td>
<td>b. colonies and clusters with an abnormal ratio of clusters to colonies (&gt;15:1)</td>
<td>19</td>
<td>10</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>c. high colony incidence (&gt;10 colonies/10^6 BM cells)</td>
<td>21</td>
<td>11</td>
<td>76</td>
</tr>
</tbody>
</table>

* BM cells, Ficoll-Hypaque-separated bone marrow cells.
**IN VITRO CLONAL GROWTH PATTERNS IN CHILDHOOD ANLL**

Table 3  Correlation between high cluster incidence in cultures of leukemic bone marrow cells and complete remission rate for 189 patients with childhood acute nonlymphocytic leukemia at diagnosis

<table>
<thead>
<tr>
<th>Growth pattern</th>
<th>No. of patients</th>
<th>% of total patients</th>
<th>CR rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High cluster incidence (&gt;/400 clusters/10^5 bone marrow cells)</td>
<td>24</td>
<td>13</td>
<td>46*</td>
</tr>
<tr>
<td>Other patients</td>
<td>165</td>
<td>87</td>
<td>81</td>
</tr>
</tbody>
</table>

* Significant (P = 0.004) correlation between high cluster incidence and low CR rate (compared to patients with <400 cluster incidence).

Type 3c (colonies and clusters with >10 colonies/10^5 bone marrow cells) included 11% of the patients and had a CR rate of 76%. Cluster counts ranged from 10 to 645 (median, 128), and colony counts, from 13 to 465 (median, 56).

Cultures of hematologically normal bone marrow cells from 18 children with solid tumors (without bone marrow involvement) yielded a variable number of colonies (range, 20 to 130; median, 46) and clusters (range, 100 to 450; median, 233). Cluster:colony ratios varied from 3:1 to 15:1.

Patients with large-cluster growth in culture showed a significantly (P = 0.03) lower CR rate (53%) than did other patients (CR rate = 79%) (Table 2). This association of large-cluster growth and low CR rate was maintained on both the DAT and VAD protocols. Other clonal growth patterns were not associated with significantly lower CR rates. Also, no correlations were noted between clonal growth patterns and FAB morphological types.

When the results were analyzed according to incidence of clusters (regardless of colony incidence), cultures from 24 of the 189 patients had greater than 400 clusters/10^5 bone marrow cells; 46% of these patients achieved CR. In comparison, 81% of patients with less than 400 clusters/10^5 bone marrow cells achieved CR (P = 0.004) (Table 3). On both the DAT and VAD protocols, the high cluster incidence group had significantly lower CR rates than did the remaining patients. There was no significant correlation between high cluster incidence and FAB type.

Morphological examination of the colony cells in the ANLL cultures generally revealed immature cells either resembling the original leukemic cells or showing evidence of partial differentiation. These latter cells had characteristics of either immature granulocytes (promyelocytes and myelocytes) or early monocytes. Also, many cultures showed extensive growth of macrophage-like cells. Partially differentiated cells with atypical features that could not be classified morphologically were also common. Small numbers of mature granulocytes (metamyelocytes and bands) were occasionally observed in cultures with colony growth.

 Cultures of normal pediatric bone marrow cells at Day 7 contained 30 to 40% mature granulocytes (metamyelocytes, bands, and segmented neutrophils) as well as immature granulocytes and monocyte/macrophage cells. The primary difference between normal bone marrow and the ANLL cultures was the greater percentage of mature cells and lack of blast-like cells in the normal cultures.

**DISCUSSION**

In this study, we have found a spectrum of leukemic clonal growth patterns in cultures from newly diagnosed childhood ANLL patients, resembling patterns previously observed in adult ANLL. Leukemic cells from different patients varied in cloning efficiency (i.e., frequency of clonogenic cells) and in proliferative potential (indicated by clonal size). The spectrum of growth patterns ranged from patients whose leukemic cells did not form clones in culture to those patients whose blasts formed large numbers of clusters and colonies. This heterogeneity may reflect patient-to-patient differences in the sensitivity of the leukemic cells to the colony-stimulating activity present in the culture, and also differences in the frequency of leukemic progenitor (clonogenic) cells in the blast population. The leukemic progenitor cells have extensive proliferative and self-renewal potential in vitro and are able to maintain the expansion of the blast population in vivo. The leukemic population is thus composed of a subpopulation of progenitor cells with clonogenic potential, and a larger population of terminally dividing or nondividing blast cells. Evidence for such a hierarchy of blast cells in ANLL has been found in studies of cell-cycle characteristics and immunological phenotypes of clonogenic versus non-clonogenic blast cells in ANLL (10–13).

Clonal assays may provide a valuable means to further classify childhood ANLL and possibly identify patients who may not respond to conventional chemotherapy. Moore et al. (2, 3), in their original studies of leukemic cell proliferation in adult ANLL, determined that growth patterns of the leukemic cells in agar culture were correlated with remission induction. In the present study, we have extended these studies to childhood ANLL. In particular, we have cultured pretreatment bone marrow blasts in soft agar in order to (a) characterize the clonal growth patterns of the leukemic cells and (b) determine the prognostic significance of the observed growth patterns in predicting the probability for an individual patient to achieve complete remission.

In agreement with earlier studies, we have found that clonal growth patterns of leukemic cells in childhood ANLL exhibit varying degrees of divergence from the normal bone marrow growth pattern. This divergence is evident in the number of ANLL clusters and colonies and in the cluster:colony ratio. Leukemic cells from 54% of the patients studied either did not proliferate in the culture (type 1a) or formed low numbers of clusters and colonies (type 1b). In agreement with findings in adult ANLL (3, 14, 15), we have found that patients with these clonal growth patterns have a relatively high CR rate (81% and 87%, respectively).

A second pattern of leukemic growth observed in the present study (type 2) was characterized by small clusters without colonies. These cluster-forming cells formed clones of 4 to 20 cells (Day 7) which did not continue to expand if cultured beyond 1 wk. Type 2 growth was associated with a high CR rate (75%) in the present study, as in the studies of Moore and Spitzer (2, 3). The favorable prognosis for this group may be related to the low proliferative potential of the leukemic cells in culture.

The remaining three growth patterns identified in this study included large-cluster growth (type 3a), cluster and colony growth with high cluster:colony ratio (type 3b), and high colony incidence (type 3c). Of these three groups, only large-cluster growth was associated with a significantly lower CR rate (53%). Large clusters consisted of 20 to 50 cells and were noticeably larger than the small clusters of type 2, indicating that the leukemic cells in this group had a higher proliferative potential in culture than did the small cluster-forming cells.

High colony incidence (type 3c) as originally defined by Moore et al. (2) (>10 colonies per 10^5 cultured cells) was not associated with a significantly lower CR rate in the present study. However, our data suggest that high colony incidence defined as >100 colonies per 10^5 cultured cells may be associ-
IN VITRO CLONAL GROWTH PATTERNS IN CHILDHOOD ANLL

ated with a lower CR rate in pediatric patients. Although only 9 patients were included in this category, 4 of these did not achieve CR (CR rate, 55%).

In the present study, pediatric ANLL cultures were further classified according to cluster incidence irrespective of colony incidence, with 400 clusters being the division point between high cluster incidence and low cluster incidence (400 clusters/10^5 bone marrow cells represented the maximum cluster incidence in normal bone marrow cultures). Patients with high cluster incidence were found to have a significantly lower CR rate (CR, 46%; \( P = 0.004 \)) than did other patients.

These results indicate that two patterns of cluster growth, based on cluster size (i.e., large clusters) and cluster frequency (high cluster incidence), were significantly associated with failure to achieve CR. The association between these growth patterns and low CR rates was maintained for patients treated with two different induction protocols (DAT and VAD).

Considering the sensitivity of these growth patterns in identifying patients who fail to achieve CR, the large-cluster pattern identified 9 of 45 (22%) patients who were nonresponders, while the high-cluster incidence pattern identified 13 of 45 (28%) nonresponders. Together, these two growth patterns identified 42% (19 of 45) of the nonresponding patients; 3 of 22 patients belonged to both the large-cluster and high-cluster incidence groups.

To investigate the relationship between colony growth and other clinical factors, a multivariate logistic regression analysis was performed to consider the correlation of the following factors with CR: clonal growth pattern; morphology (FAB type); age (under versus over 2 yr); platelets (under versus over 100,000); hemoglobin (under versus over 12%); WBC count (under versus over 100,000); splenomegaly; fever (>101°F); lymph-node involvement; and induction therapy. Induction therapy was previously found to be significantly correlated with CR rate. When this variable was adjusted for and the remaining factors considered, the large-cluster and high-cluster incidence growth patterns were found to be independently correlated with low CR (\( P < 0.05 \)). None of the other clinical variables listed above were independently correlated with remission induction after adjusting for induction therapy.

With regard to the morphology of the cells in the ANLL cultures, we have found that the cells were generally either blast-like cells, partially differentiated (including morphologically atypical cells), or macrophage-like. The abnormal nature of cell differentiation in these cultures was evident when compared to cultures of normal bone marrow, which showed 30 to 40% mature granulocytic cells.

The present results indicate that considerable heterogeneity exists among childhood ANLL patients in the ability of their leukemic cells to proliferate in agar culture. Furthermore, two parameters of in vitro growth (high cloning efficiency and high proliferative potential) appear to have prognostic significance and to be associated with resistance to cytotoxic chemotherapy. However, the finding of patients in both reduced growth (good prognosis) and excessive growth (poor prognosis) categories whose clinical course does not conform to the in vitro prediction indicates the complexity of the factors which determine clinical response. Further study of these factors, especially through the use of in vitro assays to detect drug sensitivity or resistance to individual chemotherapeutic agents, should be valuable in improving the correlation between in vitro results and clinical response. Such studies should permit the identification of subsets of patients who have a high probability of responding poorly to standard chemotherapy, and enable the design of more effective therapy for these patients. In this way, the clonal assay may be a useful supplement to other methods which can potentially identify childhood ANLL patients with poor prognosis, such as immunological and cytogenetic marker studies.

ACKNOWLEDGMENTS

The authors thank Nancy A. Ruthenberg for excellent technical assistance and Suzan Ingber for skillful secretarial assistance in preparing this paper.

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