DNA Histogram Analysis of Human Hemopoietic Cells

By B. Barlogie, G. Spitzer, J. S. Hart, D. A. Johnston, T. Büchner, J. Schumann, and B. Drewinko

The proliferative activity of human neoplasms may be an important determinant for therapeutic management. The advent of automated flow-through systems measuring cellular DNA content by means of fluorescence has considerably facilitated the analysis of cellular kinetics. Using a pulse cytophotometer ICP-11 (Phywe Co., Göttingen, Germany), three different fluorescent staining techniques for DNA histogram measurement on human hemopoietic cells were tested: mithramycin, ethidium bromide, and a combination of ethidium bromide and mithramycin. Employing the tritiated thymidine labeling index as reference standard for comparison with the DNA histogram-derived S-phase fractions, linear correlations were obtained using ethidium bromide alone and ethidium bromide in combination with mithramycin as staining techniques. The fluorescence intensity was increased fourfold to fivefold by the use of the two-dye combination, resulting in a substantial decrease in the coefficient of variation of DNA histograms to 1.5%–2%. This augmented histogram resolution is an important condition for detecting small-degree numeric chromosomal aberrations and discrete drug perturbation effects.

CONVINCING EVIDENCE has been accrued that, compared to normal hemopoietic tissue, leukemic cells have a smaller growth fraction and a longer generation time.1,2 This finding is reflected by the lower labeling index of leukemic bone marrow cells, as opposed to normal myeloid precursors.3 Recently, several investigators have demonstrated the prognostic value of a pretreatment tritiated thymidine labeling index (LI) for subsequent response to induction treatment.4 In particular, Hart et al.5 have shown, for three different combination drug regimens for remission induction of previously untreated adult patients with acute leukemia, that a blast LI > 9% is a favorable prognostic factor independent of age. This conclusion is in keeping with observations in tissue culture and in animal systems that most chemotherapeutic agents act more effectively on rapidly proliferating cells. Attempts have been made to manipulate the proliferative characteristics of the leukemic cell population by in vivo drug perturbation. Thus, using a pulse dose of 1-β-D-arabinosylcytosine (Ara-C) in patients with acute leukemia, Lampkin et al. have documented cell synchronization and/or recruitment by triggering quiescent cells into the proliferating compartment.6 Such drug-induced increase of the LI may condition patients presenting with a low LI for a better response to induction chemo-

From the Departments of Developmental Therapeutics, Biomathematics, and Laboratory Medicine, the University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas, and the University of Münster, Münster, Germany.

Submitted November 28, 1975; accepted April 19, 1976.

Supported in part by Grants CA-05831, CA-11520, and CA-14528 from the National Cancer Institute.

Address for reprint requests: Barthel Barlogie, M.D., Department of Developmental Therapeutics, University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Avenue, Houston, Texas 77030.

© 1976 by Grune & Stratton, Inc.
therapy. This kinetic treatment approach requires frequent monitoring and rapid availability of cell kinetic data, so that cytocidal chemotherapy can be administered at the time of the peak LI of the leukemic population. With the development of new in vitro culture systems for human hemopoietic cells, analysis of differential kinetic and lethal response of leukemic and normal cells to antineoplastic agents could possibly be used to spare normal stem cell damage.

One of the reasons why cell kinetics has not yet been widely used in clinical oncology is the laborious nature of previous technology. With the advent of automated cytophotometry, high-precision measurements of cellular compounds such as DNA can be rapidly performed on a large number of cells. Since DNA content is a function of cell cycle stage, DNA histograms allow a more detailed analysis of the mitotic cycle, compared to techniques that measure the incorporation of radiolabeled nucleoside precursors into DNA. Thus, cells in G0, S, and (G2 + M) phase can readily be identified. DNA histography also allows detection of aneuploid abnormalities, independent of the proliferative activity of the malignant cell clone, whereas the capacity to proliferate in vitro is an important determinant for the yield of metaphases in routine cytogenetic techniques. However, such discrimination of aneuploid clones on the basis of DNA content depends upon the resolution of the histogram.

Recently, several flow-through cytophotometers have become commercially available for the measurement of DNA content, and a variety of fluorescent staining techniques have been reported. Most of these techniques require time-consuming procedures such as hydrolysis (Feulgen method and modifications) or RNase treatment, e.g., for ethidium bromide (EB) and propidium iodide. Krishan has recently reported that by using propidium iodide in hypotonic solution, rapid DNA histograms can be obtained which are identical to those obtained after fixation and RNA digestion. Likewise, for mithramycin (MI), which binds exclusively to double-stranded DNA, no preparative procedures are required once monodispersed cell populations have been generated.

We have worked with a Phywe pulse cytophotometer ICP 11, which was developed by Göhde and Dittrich in 1969. This instrument offers the advantage that a number of fluorescent dyes can be used by altering the wavelength of fluorescence excitation by means of different filters. For optimal excitation of the MI-DNA complex, a wavelength of 390–400 nm is required. In a comparative analysis of LI and DNA histogram-derived S-phase fractions of synchronized cultures of human lymphoma cells, we have demonstrated the reliability and accuracy of the MI staining technique for pulse cytophotometric (PCP) analysis of this particular cell line.

The purpose of this paper is twofold: (1) to compare different staining techniques for DNA histogram analysis of hemopoietic cells, validating results against the 3H-TdR LI; and (2) to present several examples of the utility of high resolution DNA histography. Selecting the LI as reference standard for comparison with DNA histogram-derived S-phase compartment size, MI has proved unreliable. A linear correlation between these two parameters was obtained when hemopoietic cells were stained with EB alone or with EB and MI.
in combination. Compared to MI or EB alone, the two-dye technique led to a substantial increase in fluorescence intensity, thus providing high resolution DNA histograms with a coefficient of variation reduced to 1.5%–2%. Employing these two fluorochromes in combination, we show that discrete compartment changes in DNA histograms of hemopoietic cells can be detected. Thus, drug perturbation effects and numeric chromosomal aberrations can be identified.

**MATERIALS AND METHODS**

**Sample Processing**

Bone marrow aspirates were obtained from 35 patients with acute leukemia using preservative-free heparin as anticoagulant. The per cent blast cells ranged from 80% to 100%. Immediately after aspiration, the specimens were incubated with $^3$H-TdR (5 μCi/ml; S.A. = 6.7 Ci/mM), for 1 hr at 37°C. Erythrocytes were removed by Hypaque-Ficoll (HF) sedimentation (density = 1.078 g/cu cm, 1000g for 15 min at 4°C). Interphase cells were then collected and divided into two aliquots for subsequent autoradiographic and PCP analysis. The LI was determined on 200 nucleated cells on cytocentrifuge preparations; cells with more than 5 grains overlying the nucleus were considered labeled. For PCP analysis, the remaining aliquot of the HF-buffy coat was washed, resuspended in 0.9% NaCl, and subsequently fixed in 70% ethanol.

Leukapheresed peripheral blood, and in three instances also bone marrow, from 15 patients with leukemia were cultured in alpha medium supplemented with glutamine, L-asparagine, L-serine, and 20% fetal calf serum, at a cell density of 2 x 10⁵ cells/ml in a 5% CO₂ humidified atmosphere at 37°C. In vitro proliferation was studied without exogenous stimulus, with 20% leukocyte-conditioned medium, and in the presence of Difco-M PHA (0.005 ml per 2 x 10⁵ cells). DNA histogram and LI determinations were performed daily for 7 days. Cells were harvested and incubated with $^3$H-TdR (5 μCi/ml; S.A. = 6.7 Ci/mM) for 60 min at 37°C. One aliquot of each sample was processed for autoradiography (cytocentrifuge preparation). The LI was determined scoring 200 nucleated cells. For PCP, the remaining aliquot was washed and resuspended in 0.9% NaCl, and subsequently fixed in 70% ethanol.

**Drugs**

MI was kindly provided by Pfizer Labs, New York, N.Y. EB was obtained from Serva, Heidelberg, Germany. PHA was purchased from Difco, Detroit, Mich.; fetal calf serum from Gibco, Santa Clara, Calif.; alpha medium from Flow Labs, Rockland, Md.

**Staining Procedures for PCP Analysis**

Ethanol-fixed cells were centrifuged at 1000 g for 2 min and stained by one of the following techniques: (1) 10 ml of MI 50 μg/ml with 7.5 mM MgCl₂ and 12.5% ethanol (pH = 5.5) for 5 min; (2) 10 ml of EB 25 μg/ml (in 0.1 M Tris buffer and 0.6% NaCl, pH = 7.4) for 15 min, and (3) a combination of EB and MI. The latter procedure involved staining cells initially in 5 ml of EB 25 μg/ml (in 0.1 M Tris buffer with 0.6% NaCl, pH 7.4) for 10 min; subsequently, 5 ml of MI 50 μg/ml (containing 7.5 mM MgCl₂ and 12.5% ethanol) were added, resulting in final concentrations of 12.5 μg/ml of EB and 25 μg/ml of MI; after 5 more min, the sample was measured in the pulse cytophotometer. Routinely, 30,000-50,000 cells were measured for each DNA histogram. Previous standardization experiments had shown that coincidences of fluorescent cells passing simultaneously through the measuring chamber were negligible below a cell concentration of 5 x 10⁵ cells/ml, generating less than 500 fluorescent signals/sec. When the pulse rate indicated on the instrument exceeded 500/sec, the sample was further diluted with MI, EB, or an equivolumetric solution of the two-dye stock solution, respectively, so that EB = 12.5 μg/ml and MI = 25 μg/ml. Dye concentrations for MI, EB, and EB-MI were selected on the basis of previous publications.20,22,23,30,33

Unstained fixed cells could be stored at 4°C for 6 wk. For MI, EB, and EB-MI-stained cells, no change in the histogram configuration was observed over a 2-hr period at room temperature. MI
and EB-MI stained cells stored for 1–2 days at 4°C showed no significant difference in DNA histogram pattern with respect to that of freshly stained cells.

Treatment of ethanol-fixed cells (after rehydration) with RNase, 0.1%, for 30 min at 37°C was performed in some instances to test whether any of the three dye techniques also stained RNA.

Histogram Evaluation

DNA histogram evaluations of bone marrow samples and in vitro cultures were performed, using the criteria of Baisch et al.36 for horizontal S-phase compartments. When the S-phase fraction was skewed, a model suggested by Andreeff37 was applied, employing the mirror image technique for G1 and (G2 + M) compartments. No significant difference between repeated measurements of the same sample was observed; this confirmed the precision of the instrument.33 When split samples were measured, the standard deviation for the compartment distribution of cells in G1, S, and (G2 + M) fractions did not exceed 2%.

RESULTS

Technology

A comparative analysis of DNA histogram-derived S-phase fractions with in vitro LI determinations was performed on bone marrow cells of eight patients with acute leukemia, using MI as the DNA label (Fig. 1). Values scattered considerably, with a multiple correlation coefficient (r) of 0.607 and an error mean square (EMS) of 4.07. PCP-determined S-phase compartment size exceeded LI values for the majority of samples. The coefficient of variation (CV) of the G10 peak of the DNA histograms ranged from 7% to 10%. When EB was used on the remaining aliquots of the same fixed samples, a linear relationship was observed with r = 0.994 and EMS = 1.2. Treatment with RNase 0.1% for 30 min at 37°C did not change the histogram configuration, the compartment distribution, or the CV. The linear correlation between PCP-determined percentages of S and LI was reconfirmed for the EB analysis by addition of nine more bone marrow samples (r = 0.967, EMS = 1.888), giving a CV of the G10 compartment of 5%–10% with a median of 7%.

The two-dye staining technique (EB–MI) was employed on another 18 consecutive bone marrow specimens. A marked increase in fluorescence intensity and a decrease of the background fluorescence were observed. Thus, when the pulse cytophotometer was adjusted so that the G10 peak of cells stained with EB or MI alone appeared in channel 20, the G10 peak of EB–MI stained cells was recorded in channels 85–90. This change reflected an increment in fluorescence intensity by a factor of four to five. Compared to each fluorochrome
Fig. 2. Correlation between LI (3H-TdR) and S-phase fraction (PCP) of human bone marrow cells from 18 patients with acute leukemia, using EB and MI in combination for DNA histogram analysis. The staining procedure of ethanol-fixed cells involved 10 min staining with EB 25 μg/ml (with 0.1 M Tris buffer and 0.6% NaCl, pH 7.4); subsequently, an equal volume of MI 50 μg/ml (with 7.5 mM MgCl₂ and 12.5% ethanol) was added, so that the final dye concentrations were EB, 12.5 μg/ml and MI, 25 μg/ml. After 5 min more, the sample was measured in the pulse cytophotometer ICP 11. A fourfold to fivefold increase in fluorescence intensity was observed, compared to either EB or MI alone. A linear correlation between percentages of LI and S (PCP) was obtained (r = 0.968, EMS = 2.634).

alone, the CV of the G₁₀ compartment was considerably improved, ranging from 1.5% to 5%, with a median of 3%. As with EB alone, a linear correlation between S-phase fraction and LI was obtained, with r = 0.968 and EMS = 2.634 (Fig. 2).

The EB-MI combination was further studied utilizing in vitro cultures of leukemic peripheral blood and bone marrow (Fig. 3) with a total of 76 specimens from 15 patients, harvested at different time intervals after initiation of culture. A linear correlation between PCP-determined S-phase fraction and LI, with a narrow distribution, was found (r = 0.969) in a fashion similar to that of the analysis of fresh bone marrow specimens. In three instances, both MI and EB-MI staining techniques were applied to split fractions of the same fixed sample. Figures 4 and 5 show representative examples of the comparison be-
between DNA histograms obtained after MI and EB–MI staining techniques. There was a significant difference in the CV of the G1/0 peak, with values of 2% and 5% in the case of EB–MI staining, compared to 5% and 8%, respectively, for MI alone. In addition, compartment distributions differed to a considerable extent in the example shown in Fig. 5. The simultaneously determined LI in these two examples were in excellent agreement with the S-phase fraction after EB–MI staining.

Figure 6 shows a series of DNA histograms (EB–MI combination technique)
Figure 6. Sequential DNA histograms of AML bone marrow cells in liquid culture: (A) without stimulus; and (B) with PHA 0.005 ml Difco-M/2 x 10⁴ cells. In the absence of PHA, no significant change in the histogram pattern can be observed. In the presence of PHA, however, a marked increase in cells in S- and (G₂ + M)-phase compartments with a maximum on day 5 can be noticed. The G₁₀ peak (channel 30) shows a broadening and asymmetry of the descending (right) slope on day 4 of PHA culture; the peak of the 4C DNA complement mode is shifted beyond channel 60. This phenomenon is no longer seen on day 5. (The cells were stained with ER and MI; 30,000–50,000 cells were measured for each DNA histogram).

Clinical Application

In the following part, we present some preliminary observations, which illustrate the potential application of DNA histography to clinical problems. The high resolution EB–MI staining technique was employed in these studies.

Figure 7 shows a series of DNA histograms of both bone marrow and peripheral blood of a patient with poorly differentiated lymphocytic lymphoma. On presentation, 88% lymphoma cells were detected in the peripheral blood with a mitotic index of 0.1%. Interestingly, on DNA histogram analysis (Fig. 7A), the majority of the peripheral blood cells (84%) were in the 4C DNA complement range of (G₂ + M) cells. Similarly, the bone marrow with a lesser infiltrate of lymphoma cells (35%) also showed an increase of the (G₂ + M) compartment (33%). Routine cytogenetic screening detected only two cells that had a diploid karyotype. However, employing the technique of premature
Figure 7. Sequential DNA histograms of bone marrow and blood of a patient with malignant lymphoma in leukemic phase (poorly differentiated lymphocytic type). Ethanol-fixed cells were stained with EB and MI; 30,000–50,000 cells were measured for each DNA histogram. The slashed areas approximate the tetraploid lymphoma cell population. Notice the close correlation between the percent tetraploid cells (PCP) and the fraction of lymphoma cells identified morphologically on May-Grunwald-Giemsa-stained smears. After 3 mo of intensive combination chemotherapy, the bone marrow nearly cleared of lymphoma cells, whereas the peripheral blood still contained a considerable fraction of malignant cells. The increase of lymphoma cells in the bone marrow in (B) may be due to contamination with peripheral blood.

Figure 8 shows a series of DNA histograms of bone marrow and blood cells of a patient with acute myelogenous leukemia. Two peaks in the G10 range of the DNA histogram were observed. To identify the ploidy of these peaks, diploid chronic lymphocytic leukemia cells were mixed with this patient's samples prior to staining with EB and MI in combination. The right peak in both bone marrow and blood of this patient coincided with the diploid peak (not shown). Again, cytogenetic analysis revealed a small number of only diploid karyotypes, but no aneuploidy. However, PCC analysis documented the presence of cells with 44 chromosomes. Ultrastructural examination demonstrated the presence of nuclear blebs in the blast cells. These blebs have been reported previously to be associated with leukemic cytogenetic abnormalities.40 Residual normal myeloid progenitor cells were detected by in vitro agar culture.41

Recently, we have initiated a study designed to analyze the incidence and time course of cell synchronization and/or recruitment induced by a single
Fig. 8. Sequential DNA histograms of bone marrow and peripheral blood of a patient with AML before and after an i.v. push injection of Ara-C 200 mg/sq m. Ethanol-fixed cells were stained with EB and MI. 30,000-50,000 cells were measured for each DNA histogram. To identify the ploidy of the two peaks in the 2C range of the histograms, diploid chronic lymphocytic leukemia (CLL) cells were admixed to aliquots of the patient’s samples prior to the staining procedure. In the resulting DNA histograms (not shown), the right peak in both bone marrow and blood coincided with the diploid (CLL) peak. Consequently, the slashed area approximates a hypodiploid leukemic blast cell population with a G1 peak at X1 and a (G2 + M) peak at X2. The fraction of these cells with an abnormal DNA content correlates with the percentage of blast cells on morphological examination. The peak in channel 30 represents the residual normal diploid population. Notice the consistency of the histogram configuration over a 3-day period with distinct peaks at X1 and in channel 30.

intravenous push dose of Ara-C 200 mg/sq m. Figure 9 shows a series of bone marrow DNA histograms of a patient with acute undifferentiated leukemia (> 95% blast cells) prior to and 4, 24, and 72 hr after a pulse dose of Ara-C. Compared to the pretreatment compartment distribution, the (G2 + M) phase fraction had decreased at 4 hr (from 2% to < 1%). Twenty-four hours after administration of Ara-C, a cohort of 40% of cells was seen in early S phase, with a skewness of the lower descending (right) slope of the G1 peak. At 72 hr, the S-phase compartment showed a configuration with 20% cells equally distributed between the 2C and 4C DNA complement.

DISCUSSION

The S-phase fraction of the DNA histogram is defined as a cohort of cells with a DNA content higher than the 2C DNA complement of diploid G1 phase cells and less than the 4C DNA complement of (G2 + M) phase cells. The tritiated thymidine LI reflects the percentage of cells synthesizing DNA. Thus, both the PCP-determined S-phase fraction and percentage of LI should be identical.
under unperturbed conditions. We have evaluated three different DNA staining techniques for their usefulness in obtaining DNA histograms of human hemopoietic cells, and have tested the validity of this determination by using the $^3$H-TdR LI as the reference value of S-phase cells.

MI is a DNA-specific fluorescent dye that complexes with native DNA. Although the exact mechanism of dye-DNA interaction has not yet been elucidated, there is evidence of base specificity for guanine, and Mg$^{2+}$ ions are required in an equimolar concentration to mithramycin for maximum interaction. Our previous study utilizing a lymphoid cell line has shown a close correspondence between LI and PCP-determined S-phase fraction. When applied to hemopoietic human cells, a marked discrepancy between DNA histogram-derived S-phase fractions and simultaneously determined LI is found. Thus, under the conditions described, this dye does not meet the criteria which we have chosen to assess the validity of DNA-staining techniques.

EB is an intercalating agent, which preferentially binds to double-stranded DNA, but also to RNA. In this study, a good agreement between LI and
PCP-determined S-phase fraction values has been observed, even without the use of RNase treatment.

When EB and MI were used in combination, a fourfold to fivefold increase in fluorescence intensity and a decrease of the background fluorescence was noted. Thus the DNA histogram resolution was substantially improved and the CVs of the G_{10} peak were reduced to minimum values of 1.5%. The mechanism by which augmentation of fluorescence emission occurred is not clear at present. The maximum level of fluorochrome binding is 0.1 molecules per nucleotide for MI and 0.25 for EB. Assuming different binding sites for the two fluorochromes, the increase in fluorescence intensity may be due to additive binding. Also, energy transfer may be involved, as has been suggested for the combination of EB and Hoechst 33258 by Berkhan. The sequential staining process (EB followed by MI) rendered slightly higher resolutions compared to either simultaneous or inverse sequence. Additional RNase treatment did not improve the quality of the DNA histograms. There was excellent correlation ($r = 0.968$ for bone marrow samples and $r = 0.969$ for cultured cells) in the regression analysis of LI and S-phase percentage, documenting the validity of the combination staining technique. The total processing time (15 min for HF separation and 15 min for staining) exceeds the 5 min reported by Krishan for his rapid propidium iodide staining technique. However, this minor time delay disadvantage is amply offset by the increased resolution of the DNA histogram attained by EB–MI staining. This consistently high DNA histogram resolution is an important factor in the detection of minor changes in compartment size distribution.

A peculiar change in the histogram pattern was observed on day 4 of one PHA-stimulated culture and consisted of broadening and asymmetry of the G_{10} peak. This effect may be due to chromatin decondensation occurring in cells entering the proliferative cycle out of G_0, thus binding more fluorochrome. Such increased binding has also been observed for acridin orange and EB.

Chromosomal aberrations have been found in 40%–50% of patients with acute leukemia, and may be associated with a poorer response to chemotherapy. They are useful to monitor residual or relapsing disease. Detection of karyotype abnormality by routine cytogenetic techniques depends on the proliferative potential of the abnormal cell clone. We have presented two examples of DNA histogram abnormalities, one with a large fraction of cells in the tetraploid (G_2 + M) range and another with an extra peak in the hypodiploid range. Our suspicion of an abnormal karyotype in the absence of chromosomal aberration on routine cytogenetic screening was supported by PCC analysis and the finding of nuclear blebs in the second patient. Growth of normal myeloid progenitor cells in agar culture suggests that the normal cytogenetic karyotype and cells with a 2C DNA complement (PCP) may be derived from residual normal hemopoietic cells. In both examples, the fraction of cells with abnormal DNA content was in close agreement with the percentage of morphologically abnormal-appearing cells. Detectability of chromosomal abnormality by measurement of DNA content is independent of the kinetic behavior of the cells, but is determined by the difference in DNA content compared to normal diploid cells. Therefore, high-resolution histograms are mandatory for discrimination of small degree numeric chromosomal aberrations.
The example of drug perturbation with Ara-C revealed an increment of cells in the early S-phase range. The detection of such drug-induced changes again depends entirely on the difference in DNA content of the perturbed cells in relation to the histogram resolution. In case of a low CV, compartment changes in the G1 to S-(G2 + M) range may escape recognition.

PCP-determined DNA histograms employing EB and MI in combination can provide fast information on fluctuations of discrete compartments of the cell cycle and, thus, offer the rationale for pulses of chemotherapy scheduled on the basis of rapidly available kinetic characteristics. Since DNA histograms do not allow cell identification on a basis other than DNA content, results obtained for mixed cell populations must be interpreted with caution. Advances in cell separation methodology should enable us to analyze rapidly and sensitively cell cycle changes in both leukemic and residual normal hemopoietic cells induced by chemotherapy.48-50 Thus, differences in kinetic response of normal and leukemic cells to antineoplastic agents may be advantageously exploited to increase the lethal effects on malignant cells, while sparing normal host stem cells.

ACKNOWLEDGMENT

We gratefully acknowledge the technical skills and assistance of Ms. Susan Sumners and Ms. Marcia Lomedico. The authors wish to express their gratitude to Dr. M. J. Ahearn, who performed the ultrastructural investigations, and to Dr. W. Hittelman, who carried out the PCC analyses. We appreciate the secretarial assistance of Ms. Diane Teltschik.

REFERENCES

16. Van Dilla MA, Trujillo TT, Mullane PF, Coulter JT: Cell microfluorometry: A
DNA HISTOGRAM


43. Goldberg IH: Mode of action of anti-


47. Moore MAS, Spitzer G, Williams N, Metcalf D, Buckley J: Agar culture studies in 127 cases of untreated acute leukemias: The prognostic value of reclassification of leukemia according to in vitro growth characteristics. Blood 44:1–18, 1974


DNA histogram analysis of human hemopoietic cells

B Barlogie, G Spitzer, JS Hart, DA Johnston, T Buchner, J Schumann and B Drewinko