Effect of transferrin polymorphism on the metabolism of vitamin C in Zimbabwean adults\textsuperscript{1,2,3}


ABSTRACT
Background: Transferrin is the major iron binding protein in human plasma. In black persons, the transferrin CD phenotype has been associated with alterations in certain markers of iron status.

Objective: We studied vitamin C status in a Zimbabwean population according to transferrin phenotype because vitamin C metabolism is influenced by iron-driven oxidative stress.

Design: The study population consisted of 150 black African adults, 90 of whom were at risk of iron overload on the basis of high dietary iron content in the form of traditional beer. Transferrin phenotypes, indirect measures of iron status, and leukocyte ascorbic acid concentrations were determined. The in vitro rate of L-ascorbic acid depletion in sera from different transferrin phenotypes was investigated.

Results: The transferrin phenotype frequencies of transferrin CC and CD were 0.893 and 0.107, respectively. The iron status of transferrin CC and CD subjects was similar. After adjustment for traditional beer consumption, baseline leukocyte vitamin C concentrations were significantly higher in 16 transferrin CD subjects ($\bar{x} \pm SE$: 2.10 ± 0.34 and 2.61 ± 0.28 fmol/leukocyte in men and women, respectively) than in 134 transferrin CC subjects ($\bar{x} \pm SE$: 1.65 ± 0.11 and 1.99 ± 0.11 fmol/leukocyte in men and women, respectively; $P = 0.024$). Oral administration of ascorbic acid (2.0 g every 24 h for 48 h) led to slower rises in leukocyte vitamin C concentrations in subjects with the transferrin CD phenotype than in subjects with the transferrin CC phenotype.

Conclusion: Transferrin polymorphism may affect vitamin C status in blacks.

KEY WORDS Africa, transferrin, phenotype, vitamin C, L-ascorbic acid, iron status, scurvy, blacks

INTRODUCTION

The iron-transporting plasma protein transferrin is characterized by a genetic polymorphism (1, 2). In addition to the common transferrin type, transferrin C, anodal (B) and cathodal (D) variants have been reported (1–6). In whites, the common allele C is found almost exclusively. In US blacks, the phenotype frequency of transferrin CD is ≈10% (4). In addition, in the populations of West, central, and southern Africa, allele frequencies of ≈0.05 for the transferrin D allele were reported (7). Recently, the transferrin CD phenotype was reported to be associated with variations in certain measures of iron status (8).

The prevalence of iron overload in sub-Saharan Africa is the highest in the world, with reports that up to 10% of some rural populations are affected (9). In these African populations, iron overload was attributed to high iron intake due to the consumption of traditional iron-rich alcoholic beverages brewed in nongalvanized iron utensils (9, 10). In addition, there is now evidence for a genetic influence on iron overload in Africans that is distinct from any human leukocyte antigen-linked locus (11–13).

Increased iron stores in tissue result in accelerated oxidative catabolism of ascorbic acid, contributing to ascorbic acid deficiency (14, 15). L-Ascorbic acid (vitamin C) provides in vivo antioxidant protection primarily as an aqueous phase peroxyl and oxygen radical scavenger (16). Furthermore, vitamin C is involved in the formation of collagen and, hence, in new bone growth (17). Therefore, chronic vitamin C deficiency may lead to osteoporosis (18). In Africa, the epidemiology of scurvy (ie, severe ascorbic acid deficiency) is similar to that of severe iron overload and scurvy is often accompanied by osteoporosis (18).
Vitamin C stability may be influenced by genetic factors. It was shown that the haptoglobin (Hp) polymorphism is a determin-ant factor in the serum vitamin C concentration in whites (19). This finding was attributed to phenotype-dependent differences in iron-driven oxidative stress. In the present study, we wished to determine whether the transferrin polymorphism has an effect on the metabolism of ascorbic acid in black persons. Because leukocyte vitamin C concentrations reflect general tissue storage (20), leukocyte vitamin C concentrations of subjects with different transferrin phenotypes were compared. Furthermore, the in vivo stability of vitamin C and the rate of L-ascorbic acid depletion in serum samples in vitro were compared among subjects with different transferrin phenotypes.

SUBJECTS AND METHODS

Subjects

One hundred fifty black African adults from rural Zimbabwe were enrolled in the present study. Sixty-two subjects were husband and wife pairs with a history of traditional beer consumption. The remaining 88 subjects (45 male and 43 female) were members of 5 family pedigrees that were selected on the basis of an index subject with increased hepatocellular iron identified after liver biopsy. The liver biopsies were performed as part of a routine, clinical evaluation in different parts of the country. Subsequently, we traced these iron-loaded subjects to their homes and evaluated their relatives.

We estimated the lifetime traditional beer consumption of the subjects through interviews conducted by health personnel who were fluent in local languages and knowledgeable of local customs. Each subject was asked to estimate his or her weekly consumption of traditional beer in liters. The result was multiplied by 52, and then multiplied again by the number of years the subject had been drinking to give an estimated lifetime total of traditional beer consumption (12). In this community, the staple diet is maize meal porridge, which is eaten with various side dishes, including meat or vegetable stews mixed with tomatoes and onions. This diet was reported to be generally nutritionally adequate, with few of the micronutrients having a mean lower than what is recom-mended in the US recommended dietary allowances (RDA) (21). Vitamin C intake of 75% of the RDA values was reported in this population (22). All of the participants were examined by 1 of 2 study physicians to determine their current state of health. The Medical Research Council of Zimbabwe approved the study, and informed consent was obtained from all subjects.

Collection and analysis of blood samples

Study participants were asked to abstain from drinking any alco-holic beverages for ≥24 h before blood collection and to continue to abstain during the study period. Fasting, morning venous blood samples were drawn from each subject into K3-EDTA–coated and clotted tubes for 3 consecutive days to determine leukocyte ascorbic acid content, transferrin and haptoglobin typing, and indirect measures of iron status.

Each subject received 2.0 g vitamin C in tablet form, which they consumed orally 24 h before the second phlebotomy and 24 h before the third phlebotomy. A Coulter T890 analyzer (Coulter Electronics, Hialeah, FL) was used to measure full blood counts. Transferrin phenotyping was conducted via starch gel electrophoresis according to the method of Smithies (2). Haptoglobin phenotype was determined by using starch gel electrophoresis of hemoglobin-supplemented serum, followed by peroxidase staining (23). Serum iron concentration and total-iron-binding capacity were determined by methods modified from those recommended by the International Committee for Standardization in Hematol-ogy (24, 25). Transferrin saturation was calculated by dividing serum iron by the total-iron-binding capacity and expressed as a percentage. Serum ferritin concentration was measured with the Spectroferritin enzyme immunoassay (Ramco Laboratories, Houston). Soluble serum transferrin receptor concentrations were measured with the Quintikine enzyme immunoassay (R & D Systems, Minneapolis). Total protein and albumin were measured by using conventional colorimetric methods with a Cobas Bio auto-analyzer (Roche Diagnostic Systems, Montclair, NY). Serum protein electrophoresis was conducted by using standard cellulose acetate electrophoresis (Helena Laboratories, Beaumont, TX).

Leukocyte ascorbic acid concentrations

Leukocyte ascorbic acid concentrations were measured by a method modified from Demenson and Bowers (26). White blood cells were separated from K3-EDTA whole blood by density gra-dient centrifugation (1200 × g for 15 min at 20°C) by using 1% carboxymethylcellulose. The leukocyte fraction was homogenized in 5% trichloroacetic acid and the mixture was centrifuged at 1200 × g for 15 min at 20°C. The supernatant fluid was reacted with 0.6% copper (Cu II) sulfate solution, and 2% acidic 2,4-dinitro-phenylhydrazine was added to the mixture. The absorbance of the resultant red bis-hydrazone was measured at 520 nm (26).

L-Ascorbic acid change in vitro

In a subgroup of 44 subjects with the same haptoglobin pheno-type, Hp 2-1 (35 and 9 subjects with transferrin CC and CD phenotypes, respectively), we measured the in vitro vitamin C changes and compared the findings with the in vivo vitamin C loading experiments. Haptoglobin phenotype was described as influencing serum vitamin C stability in white persons (19, 27). In addition, L-ascorbic acid rates of change in vitro were measured in the sera of 74 healthy individuals (53 and 21 subjects with transferrin CC and CD phenotypes, respectively; all Hp 2-1 phenotype) with low dietary iron, who were described previously (8). Initial L-ascorbic acid concentrations were measured with the Hitachi 911 analyzer (Roche Diagnostics, Mannheim, Germany) by using a colorimetric ascorbate oxidase method. Vита-min C (Fluka Chemie AG, Buchs, Switzerland) was then added to the serum to increase its concentration to 570 μmol/L. The serum was then incubated at 37°C for 6 h, and the L-ascorbic acid concentration was measured at 2-h intervals.

Statistical analysis

Values are expressed as means ± SEs or as geometric means and the SE range when appropriate. Statistical comparisons of vitamin C and indirect measures of iron status according to transferrin phenotype and sex were done by analysis of variance with adjustment for estimated traditional beer consumption. The effect of vitamin C administration on leukocyte ascorbic acid concentrations according to transferrin phenotype and sex was determined by repeated-measures analysis of variance with adjustment for estimated traditional beer consumption and by three-factor analysis of variance. Values were log transformed because the values for serum ferritin concentration and estimated traditio-nal beer consumption were followed by skewed distributions.
The Student’s t test was used to compare the in vitro hourly loss of L-ascorbic acid according to transferrin phenotype. Multivariate logistic regression analysis was used to test for the effect of age; sex; transferrin phenotype; albumin; α-1–, α-2–, β–, and γ-globulins; transferrin saturation; serum iron; and haptoglobin concentrations on in vitro vitamin C stability.

RESULTS
During the physical examination, one index subject with proven iron overload showed features of scurvy and osteoporosis (eg, gingivitis, swelling gums, loose teeth, and tender vertebrae). None of the patients had diabetes mellitus or tuberculosis. The median of lifetime traditional beer consumption was 3725 L (interquartile range: 0–16630 L). Ninety of the 150 subjects had a history of lifetime traditional beer consumption was 3725 L (interquartile range: 0–16630 L). Ninety of the 150 subjects had a history of consuming ≥1000 L of traditional beer in their lifetimes.

One hundred thirty-four subjects (89.3%) were classified as having the transferrin CC phenotype and 16 subjects (10.7%) were classified as having the transferrin CD phenotype. This corresponded to an allele frequency of 0.947 for transferrin C and 0.053 for transferrin D in the population. We did not find homozygous transferrin DD cases. The haptoglobin phenotype distribution in the population was as follows: Hp 1-1, 57; Hp 2-1, 55; Hp 2-1 modified, 9; and Hp 2-2, 26. Three subjects were anhaptoglobinemic and were classified as having the phenotype Hp 0-0.

The results for leukocyte vitamin C concentration, serum iron concentration, total-iron-binding capacity, transferrin saturation, serum ferritin concentration, and serum transferrin receptor concentration according to transferrin phenotype and sex are shown in Table 1. The baseline leukocyte vitamin C concentrations were significantly higher in the subjects with the transferrin CD phenotype than in subjects with the transferrin CC phenotype. Total-iron-binding capacities, serum ferritin concentrations, and serum soluble transferrin receptor concentrations were not significantly different according to transferrin phenotype, whereas serum iron concentrations and transferrin saturations tended to be lower in subjects with the transferrin CD phenotype (NS).

After the administration of vitamin C, changes in leukocyte vitamin C concentrations differed significantly according to transferrin phenotype. Subjects with the transferrin CD phenotype had a slower change in vitamin C concentrations than did subjects with the transferrin CC phenotype (Table 2).

The results of the stability of vitamin C in serum in vitro according to transferrin phenotype are shown in Table 3. After vitamin C was added to serum, the rate of L-ascorbic acid depletion was significantly higher in subjects with the transferrin CC phenotype than in subjects with the transferrin CD phenotype. In a multivariate logistic regression model, we observed that only transferrin phenotype was a predictive factor for in vitro ascorbic acid loss ($P = 0.0001$), whereas other variables (age; sex; albumin; α-1–, α-2–, β–, and γ-globulins; transferrin saturation; serum iron; and haptoglobin) did not significantly predict loss.

<p>| TABLE 2 | Leukocyte vitamin C before and after oral administration of 2 g vitamin C according to transferrin phenotype |</p>
<table>
<thead>
<tr>
<th>Transferrin CC</th>
<th>Transferrin CD</th>
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<tr>
<td><strong>Men (n = 60)</strong></td>
<td><strong>Women (n = 55)</strong></td>
</tr>
<tr>
<td>Leukocyte vitamin C (fmol/leukocyte)</td>
<td>1.65 ± 0.11</td>
</tr>
<tr>
<td>24 h after vitamin C</td>
<td>2.04 ± 0.11</td>
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<tr>
<td>48 h after vitamin C</td>
<td>2.10 ± 0.11</td>
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$^1$ x ± SE. Changes in leukocyte vitamin C were significantly different between transferrin phenotypes, $P = 0.028$ (ANOVA with adjustment for traditional beer consumption).
We studied 150 black African adults, 90 of whom were at risk of developing iron overload on the basis of high dietary iron exposure. African iron overload has been attributed to an interaction between an environmental factor (dietary iron) and a possible genetic factor (11–13); however, there is no evidence of the primary hemochromatosis gene in this population. In a postmortem study, all 32 subjects with elevated hepatic iron indexes tested negative for the cysteine-to-tyrosine substitution at codon 282 (C282Y mutation) associated with human leukocyte antigen–linked hemochromatosis (28). In another study, a polymerase chain reaction analysis of DNA from 25 southern African subjects, identified by segregation analysis as having a high probability of carrying the African iron-loading gene, failed to identify any of the subjects with the C282Y mutation (29).

The observed transferrin phenotype distribution in the population we studied corresponds to distributions observed in other studies conducted in Africa (6, 7). Remarkably, no homozygous transferrin D phenotypes were observed in the adult populations studied. The absence of the transferrin DD phenotype was also reported in US black persons (4) and in 2 other African populations in Mozambique (30) and Rwanda (R Wieme, unpublished communication, 1999).

The aim of the present study was to investigate whether transferrin phenotypic variation influences vitamin C metabolism. Subjects with the transferrin CD phenotype had significantly higher baseline leukocyte ascorbic acid concentrations than did subjects with the CC phenotype. Subjects with the transferrin CD and CC phenotypes came from similar demographic and cultural settings, and it is therefore not likely that variations in dietary vitamin C intake were responsible for the differences in leukocyte vitamin C concentrations. The mean total leukocyte counts were also not significantly different between the 2 subject groups with different phenotypes, so the observed difference in baseline leukocyte ascorbic acid concentrations cannot be accounted for by differences in total leukocyte counts. Results of leukocyte vitamin C may be misleading when the leukocyte count is abnormal (31). Furthermore, there was no significant difference in iron status between subjects with the transferrin CD phenotype and subjects with the CC phenotype; thus, iron status cannot be implicated as the cause for the differences in baseline leukocyte ascorbic acid concentrations.

In addition to the basal differences in leukocyte ascorbic acid concentrations according to transferrin phenotype, the administration of 2.0 g vitamin C for 2 d was associated with significant differences by transferrin phenotype in the rate of change to leukocyte vitamin C concentrations. The uptake and utilization of vitamin C by the blood cells, oxidation by reactive oxygen species to their metabolites, renal excretion of vitamin C, and vitamin C redistribution between various body compartments may all be partly attributable to the observed leukocyte vitamin C change in vivo after a program of oral vitamin C administration (32). It is also possible that the observed differences in the evolution of leukocyte vitamin C concentrations after vitamin C supplementation may in part be genetically determined.

In the in vitro experiments, there was a 10% higher rate of vitamin C depletion in sera from subjects with the transferrin CD phenotype in sera than from subjects with the transferrin CC phenotype, which suggests that vitamin C may be more stable in serum samples obtained from subjects of the transferrin CD phenotype. In subjects with transferrin CC and CD phenotypes combined, the in vitro rate of reduced vitamin C concentrations correlated with the in vivo vitamin C change between the second and third doses of vitamin C. In the in vitro experiments, the loss of ascorbic acid in serum may be largely due to oxidation by reactive oxygen species present to form dehydroascorbic acid. In addition, these in vitro findings increase the likelihood that the differences between the transferrin variants in vivo may not be completely due to differences in dietary intake.

The higher leukocyte vitamin C concentrations in subjects with the transferrin CD phenotype might be partly explained by the lower serum iron concentrations in these subjects and associated lower iron-driven prooxidative stress. However, as mentioned previously, according to serum ferritin concentrations, iron status was similar between subjects with the transferrin CD and CC phenotypes.

It was reported that smokers have low vitamin C values (33). In the present study we did not evaluate the smoking habits of the subjects. Generally, smoking seems to be an activity of affluent members of the society. Surveys conducted in Zimbabwe showed that smoking is more prevalent in urban settings (34, 35), with 20% of males and <2% of females reporting that they smoked. The setting of our study was in rural communities of a low economic status, and there are cultural taboos against women smoking. It is probable that the
prevalence of smoking in our subjects was very low. Consequently, the effect of smoking on our findings is unlikely to be important. Nevertheless, future studies may need to be stratified according to smoking habits, in view of the fact that the population is in transition.

Our findings suggest that transferrin polymorphism might be a factor affecting the vitamin C status of blacks. Although the observed differences were not associated with a clinical deficiency of vitamin C in the population studied, it is conceivable that transferrin polymorphism plays a role in the pathogenesis of scurvy.

REFERENCES