Effects of Vitamin E in Kid Erythrocytes on Tween 20-Induced Hemolysis in vitro

Kyoko Hodate and Tatsuo Hamada

Department of Nutrition, National Institute of Animal Industry, Tsukuba Norindanchi, Ibaraki 305, Japan

(Received May 9, 1984)

Summary Red blood cells (RBC) of alpha-tocopherol sufficiency and deficiency were produced in kids fed on purified diets to investigate the relationship between RBC alpha-tocopherol levels and hemolysis induced by Tween 20. RBC alpha-tocopherol contents of vitamin E-deficient kids were non-detectable from 4 weeks, and percentages of hemolysis increased to over 70%. A highly significant decrease in hemolysis was noted when vitamin E was added to the diet. Tween 20 hemolysis values were negatively correlated with RBC tocopherol levels in kids. The concentration of RBC alpha-tocopherol required to prevent the hemolysis was found to be 0.6 μg/ml packed cells.

Key Words Tween 20, hemolysis, vitamin E, kid, red blood cells

Evaluation of vitamin E in ruminant nutrition has received considerable attention in recent years (1, 2). Tocopherol found in red blood cells (RBC) is localized exclusively in the membrane fraction (3). It is well established that vitamin E in cellular, and subcellular membranes are the first line of defence against peroxidation of vital phospholipid (4). RBC are known to be destroyed in the tocopherol-deficient state by exposure to oxidative agents such as hydrogen peroxide (5). However, in domestic animals hemolytic methods in vitro have given inconsistent and unreliable results (6).

Tween 20 (polyoxyethylene sorbitan monolaurate) is a nonionic surfactant which can cause the specific hemolysis of vitamin E-deficient RBC of several animals (7–9). Hydrogen peroxide and dialuric acid also have this effect. The hemolytic activity of polyoxyethylene-derived surfactant has been investigated (10). It can be a valuable tool for the rapid evaluation of vitamin E inadequency. The present investigation was undertaken to determine the correlation of RBC tocopherol levels with susceptibility to Tween 20 hemolysis occurring in kid RBC.
EXPERIMENTAL

Animals. The animals used were six 7-day-old Japanese native meat-type kids weighing about 1.7 kg at the start of the experiments.

Diet. The kids were divided into two groups of 3 animals each. The kids of the first group were fed on a vitamin E-supplemented diet and those of the second group were fed on a vitamin E-free diet. The composition of the experimental diets is shown in Table 1. These diets were reconstituted with warm water at a ratio of 1 to 4 (7). Vitamin E-supplemented milk contained 200 mg dl-alpha-tocopheryl acetate per kg dry matter. Daily amounts of the milk-replacer solid fed were 60 and 82 g for the first 4 weeks and the following 7 weeks, respectively.

Preparation of blood samples. Jugular blood was taken from the kids using heparinized vacutainers just before morning feeding. Whole blood was immediately used for the hemolysis of Tween 20. Heparinized blood was centrifuged at 3,000 rpm for 15 min. The RBC were washed with three 10 ml portions of physiological saline, and the final hematocrit of the RBC suspension was adjusted to about 50%, precise hematocrit measurement being carried out. Plasma and packed cells were frozen at -80°C until the determination of tocopherol contents. All analyses were made within 1 week of sample collection.

Determination of hemolytic activities. The susceptibility of RBC to hemolysis by Tween 20 was determined as shown previously (7). Twenty-five volumes of saline-phosphate buffer (pH 7.4) containing 0.5 mM Na-EDTA was added to 1 volume of heparinized blood and the mixture was centrifuged at 3,000 rpm for 10 min. The cell pellet was resuspended with the same volume of the above buffer. A 0.5 ml aliquot of the cell suspension was mixed with 0.5 ml of 5% (v/v) Tween 20 in 0.9% saline and incubated at 37°C for 15 min. Immediately after the incubation, 2.5 ml of ice-cold saline-phosphate buffer was added to the incubation mixture, followed by

Table 1. Composition of vitamin E-deficient diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried skim milk</td>
<td>88.0</td>
</tr>
<tr>
<td>Lard</td>
<td>10.0</td>
</tr>
<tr>
<td>Lecithin</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>0.6</td>
</tr>
<tr>
<td>Micro mineral mixture</td>
<td>0.2</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a One kilogram of diet contained: vitamin A, 10,000 I.U.; vitamin D, 2,000 I.U.; vitamin B12, 5 mg; vitamin B6, 35 mg; vitamin B6, 2.5 mg; niacin, 30 mg; Ca-pantothenate, 54.5 mg; and choline-Cl, 288 mg. b One gram of micro mineral mixture contained: Mn, 100 mg; Fe, 100 mg; Cu, 200 mg; Zn, 120 mg; and I, 2.0 mg.

centrifugation. The optical density of the supernatant was measured at 540 nm. Percentage hemolysis was calculated by dividing the above optical density by the optical density of the completely hemolyzed sample and multiplying by 100.

_Tocopherol assay._ Tocopherol analogues existing in plasma and RBC were determined by high-performance liquid chromatography according to the method shown previously (11, 12).

**RESULTS**

No significant difference was observed in weight gains of kids between the vitamin E-supplemented and vitamin E-deficient groups over the 11-weeks experimental period.

Of the analogues of tocopherols, only alpha-tocopherol was found in plasma and RBC. Changes in plasma and RBC levels of alpha-tocopherol in each dietary group during the 11-weeks test period are presented in Table 2. The alpha-tocopherol contents for the plasma from vitamin E-supplemented kids rose sharply to 6.48 µg/ml after 1 week and remained relatively steady at the above level thereafter. The mean alpha-tocopherol levels in the RBC from vitamin E-supplemented kids was 3.45 µg/ml packed cells at 1 week and decreased to 1.79 µg/ml packed cells by 11 weeks. In the vitamin E-supplemented kids the ratio of the alpha-tocopherol level in RBC to that in plasma was 0.53 at 1 week. It tended to decrease throughout the experimental period. The mean alpha-tocopherol level in plasma was 0.55 µg/ml from vitamin E-deficient kids at 1 week and maintained a relatively constant value of 0.5–0.7 µg/ml thereafter. The RBC tocopherol level

<table>
<thead>
<tr>
<th>Vitamin E state</th>
<th>Experimental period (weeks)</th>
<th>1</th>
<th>4</th>
<th>7</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma alpha-tocopherol levels (µg/ml)</td>
<td>+</td>
<td>6.48 + 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.50 + 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.45 + 0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.40 + 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.55 + 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49 + 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.64 + 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66 + 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RBC alpha-tocopherol levels (µg/ml)</td>
<td>+</td>
<td>3.45 + 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.48 + 0.17</td>
<td>2.11 + 0.51</td>
<td>1.79 + 0.33</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.74 + 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Hemolysis (%)</td>
<td>+</td>
<td>9.9 + 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6 + 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.8 + 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.7 + 5.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>17.3 + 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>77.2 + 5.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.7 + 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.9 + 3.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Means of 3 animals (±SD) denoted by a and b were significantly different (p<0.01),<br><sup>c,d</sup> (p<0.05),<br>not-detectable.
Fig. 1. Correlation of hemolysis induced by Tween 20 to RBC tocopherol levels.

from vitamin E-deficient kids was 0.74 μg/ml packed cells at 1 week but was non-
detectable from 4 weeks.

A graded response of RBC from vitamin E-supplemented and -deficient kids to
the hemolytic action of Tween 20 was observed. The results are summarized in
Table 2. At 1 week the hemolytic value in vitamin E-deficient kids was 17% and
increased to 77% by 4 weeks, and thereafter remained at about 80%. The average
value for vitamin E-supplemented kids declined from 10% at 1 week to 4% at 4 weeks
and then increased to 15% over the next 7 weeks.

Figure 1 shows the relationship between the RBC tocopherol content and 2.5%
Tween 20-induced hemolysis. The percentages of hemolysis and the RBC toco-
pherol levels showed a reciprocal relationship. The threshold of alpha-tocopherol
concentration to the rapid increase in hemolysis was 0.6 μg/ml packed cells.

DISCUSSION

In this paper we studied the relationship between RBC tocopherol levels and
the development of Tween 20 hemolysis, and found a close negative correlation
between them. When RBC tocopherol levels decreased to 0.6 μg/ml packed cells or
less, kid RBC became more susceptible to 2.5% Tween 20 hemolysis.

Ishibashi et al. (11) have reported that the ratios of tocopherol in RBC to that
in plasma differ between animal species but are relatively constant within the same
species, even if the tocopherol level is increased by supplementation. Mino et
al. (13, 14) have reported that the ratio of RBC tocopherol to plasma tocopherol is
not constant and decreases during development. Bieri et al. (15) have reported that
the distribution of tocopherol between RBC and plasma can be markedly affected
by several physiological variables and that the RBC content of tocopherol decreases
as plasma lipid increases. In our work, the alpha-tocopherol level in plasma
remained unaltered in vitamin E-supplemented kids but the level in RBC decreased,
and the alpha-tocopherol content of RBC after 4 weeks in vitamin E-deficient kids

was too small to be detected.

Ewan et al. (16) have found that vitamin E supplementation reduces erythrocyte susceptibility to dialuric acid hemolysis in lambs. Whanger et al. (17) have reported that the RBC from selenium and vitamin E-deficient sheep are not more fragile than those from sheep receiving vitamin E and selenium. Boyde (18) has reported that lambs fed on skim milk containing cod liver oil develop muscular dystrophy, although no abnormal changes are observed in peroxide hemolysis, and that after replacing cod liver oil by maize oil, no muscular dystrophy but an increased hemolysis is observed. He has concluded that the peroxide hemolysis test is not a useful measure of vitamin E deficiency in lambs and that increased peroxide hemolysis in lambs depends on the fatty acid composition of the unsaturated lipids in the diet.

Tween 20 causes hemolysis in vitamin E-sufficient cells as well as in vitamin E-deficient ones, and by proper selection of Tween 20 concentration a clear distinction between vitamin E-deficient cells and vitamin E-sufficient cells can be made (9). Rat RBC are hemolyzed with greatly diluted Tween 20 irrespective of their vitamin E status. Vitamin E-specific hemolysis of rat RBC can be induced by Tween 20 with ascorbic acid and azide (8). It has been reported that polyoxyethylene-derived surfactants have a strong tendency to autocatalytic peroxide formation in aqueous solution (19). Peroxide and free radicals are known to cause membrane damage and to induce hemolysis. Tween 20 may cause oxidative damage to membrane lipid through the formation of peroxide and free radicals during autooxidation.

REFERENCES

9) Hamada, T., Hodate, K., and Nakayama, E. (1984): Vitamin E, catalase, manganous or cobaltous ions and dithiothreitol protect against Tween 20-induced hemolysis of


