THE EFFECT OF SPLENECTOMY AND DIETARY PROTEIN ON ERYTHROCYTE SURVIVAL AND FRAGILITY IN RATS

Fumiko Hisaoka and Keizo Shiraki

Department of Nutrition, School of Medicine, Tokushima University, Tokushima, Japan
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Studies were made on the effects of splenectomy on the red cells of rats receiving various dietary levels of protein. Anemia was pronounced and the survival time of red cells (T/2 Rbc) was reduced in rats on a protein-free diet as compared with rats on standard and low protein diets.

The spleen appeared to be relatively hypofunctional in rats on low protein diets since (a) splenectomy had the least effect on increasing T/2 Rbc in rats on low protein diet and largest effect on survival of Rbc's in rats on a normal diet; (b) splenectomy had the least effect on osmotic fragility of red cells in rats on low protein diet and the most effect was shown on osmotic fragility of rats on normal diet; (c) the weight of the spleen was least in rats on a low protein diet in comparison with the other two groups.

The red cells of normal rats on a protein-free diet were most sensitive to lytic effect with lysolecithin, and Rbc's from splenectomized rats were more resistant to osmotic lysis than Rbc's from intact rats. The decrease in the T/2 Rbc and the sensitivity to a hemolytic agent (lysolecithin) of cells of rats on low protein or protein-free diet observed in this study may be related to the mechanism of anemia during inadequate protein nutrition.

It has been believed that an adequate amount of dietary protein is essential for maintenance of the normal hemoglobin level of the blood, while a diet without adequate and sufficient protein causes anemia. Delmonet et al. (5) suggested that the anemia caused by protein deficiency was hemolytic in nature and was due to structural defects in the erythrocytes. Erythrocytes which are slightly abnormal in structure may be destroyed when they pass through the reticuloendothelial system, particularly the liver and spleen (1, 6).

1 久間文子，白木啓三
The present experiments were designed to study the effect of the splenectomy on erythrocytes of rats receiving various dietary levels of protein. Normal and splenectomized rats were fed diets containing various levels of protein. The red cell survival time \((T/2\text{ Rbc})\) of the rats was measured \textit{in vivo} and the osmotic fragility of their red cells and the sensitivity of these cells to a hemolytic agent were examined \textit{in vitro}.

**MATERIALS AND METHODS**

Female Wistar rats, weighing from 200 to 250 g, were used. Animals weighing about 80 g were subjected to splenectomy at least 4 weeks before experiments. The operation was performed under ether anesthesia through a small incision in the abdominal wall. After the operation, hematocrit values were measured twice a week and when no fall in the postoperative hematocrit value was observed during the periods, the animals were considered to be free from Bartonella muris infection.

Rats were housed in individual cages at 24°C and intact and splenectomized rats were divided into 3 groups.

For 47–50 days group 1 received a balanced synthetic diet "standard protein" consisting of 20% vitamin-free casein, 44.5% \(\alpha\)-starch, 22.2% sucrose, 2% cellulose powder, 5% corn oil, 4% salt mixture (containing 99 mg of iron per 100 g of mixture) and 1% vitamin mixture. Prior to use, 1,500 IU each of vitamin A and vitamin D, and 200 mg of choline chloride were added per 100 g of food. Food consumption was recorded every day, the average daily food intake per rat of this group over a period of 42 days was 12 g (2.03 g of protein).

For 45–50 days rats of group 2 received a "low protein" diet having the same composition as the standard diet except that it contained 5% casein, 54.4% \(\alpha\)-starch and 27.2% sucrose. The average daily food intake per rat in this group was 12 g (0.51 g of protein).

Rats of group 3 received a "protein-free" diet for 40–46 days. Their diet had the same composition as the standard diet but casein was entirely replaced by \(\alpha\)-starch and sucrose without changing the 2:1 ratio by weight. The average daily food consumption per rat in this group was 7.8 g.

All the animals were first fed on laboratory stock rations. Animals in groups 1 and 2 were given synthetic diets when their weight reached 150 g and those in group 3 were given synthetic protein-free diet when their weight reached 200 g.

Erythrocyte survival time was measured after the rats were fed the synthetic diets for 3 weeks, and the procedures are as follow. Isologous, heparinized blood was taken by heart puncture from the donor rats in the three diet groups under ether anesthesia, and the blood was labeled with 20–25 \(\mu\)Ci of radioactive sodium chromate (Dainabot, Tokyo; specific activity 70.3 mCi/mg \(^{51}\text{Cr}\)) per ml of the blood. The blood was incubated with \(^{51}\text{Cr}\) at room temperature for 60 min. Incubation was terminated by adding 10 mg of sodium ascorbate per ml of blood.
The red cells were washed twice with 0.9% saline, and resuspended in the original volume with 0.9% saline. A 0.5 ml/100 g of body weight dose of the labeled blood cells was injected into the tail vein of the recipient rats. Blood samples were taken from the recipient rats at regular intervals (every 3 days) from 24 hr after the time of injection of the labeled red cells. 0.02 ml of blood was taken from the tail vein and placed into tubes containing 1 ml of water for radioactivity measurement; hematocrit value was measured at the same time. The radioactivity of the blood was determined in a well-type scintillation counter (Autogamma Spectrometer, Packard, 5000 series). The radioactivity of the blood measured 24 hr after injection was taken as 100%, and the radioactivities of samples counted on the particular day were expressed as percentages of the value in a 24-hr sample. Corrections were made for both the hematocrit value and the blood volume, which was estimated from the body weight (7).

Other routine measurements of the blood were made by standard methods, and the concentration of plasma protein was measured by the semimicro Kjeldahl method.

Osmotic fragility of the red cells was measured by a modified DACIE and VAUGHAM method (4). A series of hemolysis tubes was set up containing 10 ml of neutral saline of graded concentrations of 0.30 to 0.60% with a concentration difference between successive tubes of 0.02%. Washed red cells were resuspended in saline and adjusted to a hematocrit value of approximately 33%. 0.1 ml aliquot of this suspension was put into each hemolysis tube. After 3 hr at room temperature, the tubes were centrifuged at 1,500 rpm for 5 min and hemolysis in the tube was determined by measuring the absorption of the supernatant at 545nm. Hemolysis in the tubes was expressed as a percentage of that of a sample hemolyzed in water after the same time.

Lysolecithin (Sigma) was used to determine the sensitivity of red cells to chemical lysis. The amount of added lysolecithin (in mg) causing 50% hemolysis of 1 ml of blood in 30 sec at 24°C was taken as the hemolytic sensitivity index. The temperature coefficient of hemolysis with lysolecithin is only 1.05 between 20 and 30°C (3).

A 0.02 ml sample of heparinized whole blood was diluted to 6.0 ml with buffered saline (4 volumes of 0.95% NaCl plus 1 volume of 1/8 M sodium phosphate buffer, pH 7.4) in a round cuvette. The suspension was mixed with 0.1 to 0.2 ml of lysolecithin solution (0.25 mg/ml in ethanol) by stirring continuously with a small magnetic stirrer. Then the turbidity of the mixture was measured using a turbidimeter connected with a recorder. The red cells were thus exposed to varying concentrations of lysolecithin and the times in seconds at which 50% hemolysis was produced at each concentration were plotted on semilogarithmic paper against the concentration of lysolecithin added. The amount of lysolecithin causing 50% hemolysis in exactly 30 sec was obtained by interpolation. This value multiplied by a factor 50 was taken as the Rbc hemolytic sensitivity
index, expressed as mg of lysolecithin per ml of blood. The validity of this method was reported elsewhere (8).

After the measurements of red cell survival time were made, the rats were still kept on respective synthetic diets, and on the 45th day on the average from the beginning of the dietary controls, the rats were sacrificed under ether anesthesia by drawing blood via heart puncture. In this way about half the circulating blood could be withdrawn, and the blood thus collected was used for the tests described above.

The spleens were removed and weighed immediately after sacrificing the rats.

RESULTS

The hematologic data of intact and splenectomized rats fed various levels of protein diets are summarized in Table 1. Significant reductions (p<0.05 and p<0.001, in intact and splenectomized rats, respectively) of Rbc in rats on a protein-free diet were observed, while Rbc in rats on standard and low protein diets was identical. The concentrations of hemoglobin and plasma protein decreased depending on the levels of the protein in the diets. The hematocrit values were also identical in the rats on standard and low protein diets, and highly significant reductions (p<0.001, both in intact and splenectomized rats) were observed in rats on a protein-free diet.

The red cell survival times (T/2 Rbc) of intact and splenectomized rats are shown in Table 2. The average T/2 Rbc of rats on a low protein diet is slightly shortened, but the difference from that of rats on standard protein diet is not significant. The T/2 Rbc of rats on protein-free diet is reduced significantly (p<0.01).

Table 1. Hematologic data of the rats. Numbers in parentheses indicate number of rats/group. Values are means ± S.D. Level of significance of difference from value of corresponding group 1:

| Group | Diet and treatment | Rbc (10^6/cmm) | Hb (g/100 ml) | Ht (%) | Plasma protein (g/100 ml) |
|-------|-------------------|----------------|---------------|--------|-------------------------|
| 1     | Standard protein  |                |               |        |                         |
| Intact|                   | 716±63         | 14.3±0.9      | 41.5±2.5 | 6.43±0.16               |
| Splenectomized | 18 | 677±41         | 13.4±1.2      | 40.7±1.6 | 6.63±0.36               |
| 2     | Low protein       |                |               |        |                         |
| Intact|                   | 711±31         | 13.3±0.7*     | 40.5±2.1 | 6.04±0.32**            |
| Splenectomized | 11 | 687±36         | 13.1±0.4     | 40.8±1.0 | 5.62±0.43*              |
| 3     | Protein-free      |                |               |        |                         |
| Intact|                   | 655±47*        | 12.7±0.4***   | 36.9±1.1*** | 4.52±0.36***          |
| Splenectomized | 10 | 565±39***     | 10.8±0.7***   | 31.6±1.9*** | 5.30±0.24***           |
Table 2. Red cell survival (T/2 Rbc) and weight of spleen. Numbers in parentheses indicate number of rats/group. ΔT/2 Rbc, difference of T/2 Rbc between splenectomized and intact rats. Values are means ± S.D. Level of significance of difference from value of corresponding group: *, p<0.05; **, p<0.005; ***, p<0.001.

| Group | Diet and treatment | T/2 Rbc (Day) | ΔT/2 Rbc (Day) | Ratio of T/2 Rbc (Splenect./Intact) | Weight of spleen (g/100 g) |
|-------|-------------------|---------------|----------------|---------------------------------|---------------------------|
| 1     | Standard protein  |               |               |                                 |                           |
|       | Intact            | 16.4±3.1      | 8.5           | 1.52                            | 0.288±0.037               |
|       | Splenectomized    | 24.9±5.3      |               |                                 |                           |
| 2     | Low protein       |               |               |                                 |                           |
|       | Intact            | 15.8±2.7      | 4.6           | 1.29                            | 0.173±0.017**             |
|       | Splenectomized    | 20.4±3.7      |               |                                 |                           |
| 3     | Protein-free      |               |               |                                 |                           |
|       | Intact            | 11.3±1.5***   | 3.5           | 1.31                            | 0.181±0.025*              |
|       | Splenectomized    | 14.8±2.5***   |               |                                 |                           |

This table also shows that splenectomy increases the T/2 Rbc considerably. The ratio of the T/2 Rbc of splenectomized rats to that of intact rats differed with the level of protein in the diet, suggesting that the effect of splenectomy on the T/2 Rbc differs with the nutritional status of rats. The ratio (i.e., the influence of splenectomy on survival of the red cells) was largest in rats on standard diet. The ratio was lowest in rats on low protein diet and intermediate in those on a protein-free diet. The spleen weight was highest in rats on the standard protein diet, least in those on low protein diet, and intermediate in those on a protein-free diet. The weight of the spleen in the three groups seemed to be directly correlated with the effect of splenectomy on Rbc survival.

The osmotic fragility of the red cells is shown by the curves of hemolysis in hypotonic saline solutions in Fig. 1a and 1b. With intact rats, as shown in Fig. 1a, the low protein diet caused a shift of the curve to the right, indicating decreased fragility. As shown in Fig. 1b, the splenectomy shifted all the curves to the right of those of the corresponding groups of intact rats.

The concentration of NaCl giving 50% hemolysis of the red cells (here termed the half hemolysis rate, HHR) was obtained from these hemolysis curves, and is shown with the hemolytic sensitivity index in Table 3. With intact rats, the HHR of red cells from rats on a low protein diet is significantly less (p<0.05) than that of rats on standard diet, indicating red cells from the former are less fragile, but that of rats on a protein-free diet is not significantly different from the latter. On the contrary, with splenectomized rats, the HHR values of cells from rats on a low protein or protein-free diet were significantly higher than those of rats on standard protein diet, indicating that these cells are more fragile than the latter. Thus splenectomy caused the greatest change in osmotic fragility of cells of rats.
Fig. 1a and 1b. Hemolysis of rat red cells. 1a, hemolysis curve for the intact rat. 1b, hemolysis curve for the splenectomized rat. Points are average values. For details, see text. ●—●, standard protein; ×—×, low protein; ○—○, protein deprived.

Table 3. Osmotic fragility and homolytic sensitivity index of red cells. Numbers in parentheses indicate number of rats/group. HHR, half hemolysis rate (percentage concentration of NaCl giving 50% hemolysis of red cells). ΔHHR, difference of HHR between intact and splenectomized rats. Level of significance of difference from value of corresponding group 1:

* , p<0.05; ** , p<0.005; ***, p<0.001.

| Group | Diet and treatment | HHR   | ΔHHR  | Hemolytic sensitivity index |
|-------|--------------------|-------|-------|-----------------------------|
| 1     | Standard protein   |       |       |                             |
| Intact| (12)               | 0.491±0.018 | 0.082 | 2.095±0.254                |
| Splenectomized | (18)            | 0.409±0.017 |       | 2.167±0.254                |
| 2     | Low protein        |       |       |                             |
| Intact| (11)               | 0.459±0.019** | 0.034 | 2.084±0.284                |
| Splenectomized | (11)           | 0.425±0.018*  |       | 2.147±0.345                |
| 3     | Protein-free       |       |       |                             |
| Intact| (8)                | 0.485±0.018 | 0.054 | 1.709±0.273**              |
| Splenectomized | (10)            | 0.431±0.022*  |       | 1.532±0.262***            |
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on standard protein diet, and least in those on a low protein diet. These differences in the HHR between splenectomized and intact rats in the different groups correlated well with differences in weight of their spleens and also in the ratios of T/2 Rbc, shown in Table 2.

The hemolytic sensitivity of red blood cells to lysolecithin (here termed the hemolytic sensitivity index) is summarized in Table 3. The values for both intact and splenectomized rats are proportional to the levels of protein in the diets, being highest in the cells from rats on standard protein diet. The results indicate that red cells from rats on protein-free diet have an increased sensitivity to chemical lysis by lysolecithin.

DISCUSSION

In this study, a reduction of hemoglobin in rats on low protein and protein-free diets was observed and the mean T/2 Rbc in intact rats on standard protein, low protein and protein-free diets were 16.4 days, 15.8 days and 11.3 days, respectively. In splenectomized rats, the T/2 Rbc was longer than in intact rats, and the extent of its prolongation was proportional to the level of protein in the diet. THOMPSON et al. (9), and ULMANN and GORDON (10) measured the T/2 Rbc in rats after splenectomy using 51Cr and found that it was prolonged by approximately 2.5–2.8 days. However, they did not control the constituents of the diet but used normal stock diets. In our study, splenectomy prolonged the T/2 Rbc in rats receiving standard protein, low protein or protein-free diets by 8.5, 4.6 and 3.5 days, respectively. The ratio of the T/2 Rbc values of splenectomized to intact rats varied with the protein level of the diet: the highest ratio (1.52) was obtained in rats on standard protein diet and the lowest (1.29) in those on a low protein diet.

In this work, the differences in the weight of the spleen between the rats receiving different protein diet were found to be in good correspondence with differences in the effects of splenectomy on the T/2 Rbc value and osmotic fragility of the red cells. Namely, the weight of spleen per 100 g body weight was highest in rats on standard protein diet and the effect of splenectomy on the T/2 Rbc and the osmotic fragility were also largest in this group, while the effect was least in rats on low protein diet in which the weight of the spleen per unit body weight was lowest.

As to the hemolytic sensitivity to lysolecithin, our experiments showed that
in both intact and splenectomized rats, red cells from rats on protein-free diet were more sensitive in a fixed time to lysolecithin than those on a standard protein diet, and splenectomy did not affect the sensitivity of Rbc's to lysis with lysolecithin. It seems likely from our experiments that the sensitivity to chemical lysis reflects the nutritional status rather than the function of the spleen.

Thus it may be reasonable to assume that change in resistance of erythrocyte membrane to lysolecithin may be a factor involved in the mechanism of adaptation to protein deficiency.

On the other hand, the decreased osmotic fragility of cells from rats on low protein diet should be explained by different mechanism from the above. Starting from these results further experiments are now being performed for analysis of factors controlling erythrocyte resistance not only to hypotonicity but also to chemical hemolysis.

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