THE INFLUENCE OF INCREASED HEPATIC SEQUESTRATION AFTER SPLENECTOMY ON THE SURVIVAL AND OSMOTIC FRAGILITY OF RED CELLS IN RATS, WITH REFERENCE TO PROTEIN LEVELS IN DIETS

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Summary The sequestration of erythrocytes in rats was studied using an isologous ^51^Cr-labeled population of either normal or N-ethyl-maleimide (NEM) treated red cells. The spleen sequestered the damaged red cells selectively, while the liver compensated and overshot the sequestration for spleen after splenectomy. The sequestering response in liver increased gradually reaching a maximum level around 8 weeks after splenectomy and then declining toward the control level. These compensatory responses in liver were not observed in rats fed a low-protein diet, which indicated that the proliferative response imposed on liver by an extra work after splenectomy was not stimulated in the rats fed a low-protein diet.

Splenectomy prolonged erythrocyte survival and reduced the osmotic fragility of normal red cells, but the compensatory increase in sequestration of damaged red cells in liver did not alter the survival and osmotic fragility of normal red cells of the rat. This fact indicates that the increased sequestration of reticuloendothelial cells in liver is basically reparative, and it is impossible to compensate for the absence of the spleen because of an inability to duplicate certain anatomic features peculiar to the spleen.

The spleen is the main site of sequestration of effete red cells from circulation (1), and splenectomy leads a compensatory increase in the hepatic sequestering function. This compensatory increase in hepatic sequestration is attributed to the increased reticuloendothelial functions stimulated by the extra work imposed

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on liver after splenectomy (2). Namely, the proliferative response of Kupffer cells to foreign particles is similar to the splenic response to sequester the autologous blood cells.

When red cells are treated with an appropriate concentration of alkylating agents, such as N-ethyl-maleimide (NEM) or p-mercuribenzoate (PMB), the red cells become susceptible to selective splenic sequestration and destruction (3). After splenectomy, the treated red cells are sequestered in other reticuloendothelial cells, such as Kupffer cells.

Thus, the compensatory response of liver after splenectomy may be demonstrable by observing the changes in sequestration rate of altered red cells. Jacob et al. (2) demonstrated that the proliferative response of Kupffer cells increased gradually, achieving peak level at around 8 weeks after splenectomy. There are reports that splenectomy reduces osmotic fragility and prolongs survival time of normally circulating red cells (1, 4-6). But there has been hitherto no report of which we know indicating that the osmotic fragility and survival time of normally circulating red cells may be changed with changes in compensatory response of liver after splenectomy.

Accordingly, present experiments were designed to observe whether the changes in the hepatic sequestration of altered red cells may influence osmotic fragility and survival time of circulating red cells. For this purpose, the sequestration rate in liver at different periods after splenectomy was measured in relation to the survival time and osmotic fragility of red cells in rats. Since the proliferative response of Kupffer cells in liver may be influenced by diets (7), these parameters were also observed by comparing the rats under different nutritional conditions.

**METHODS AND MATERIALS**

**Grouping of the animals.** Commercially obtained male Wistar rats were used for the experiments. Experimental schedules and grouping of the rats are illustrated in Table 1. When the rats achieved a body weight of 200 g, they were divided into two groups according to diet; the standard protein diet group and the low-protein diet group.

The standard protein diet consisted of 20% vitamin-free casein, 44.5% α-starch, 22.2% sucrose, 2% cellulose powder, 5% corn oil, 4% salt mixture, and 1% vitamin mixture. Prior to use, 1,500 I.U. each of vitamin A and vitamin D, and 200 mg of cholin chloride were added per 100 g of food. The low-protein diet had the same composition as the standard protein diet except that it contained 5% casein, 54.4% α-starch and 27.3% sucrose.

All rats were housed in individual cages and food and water were given ad libitum.

After each group was consecutively fed each diet for two weeks, splenectomy and a sham operation were performed. The average body weights of the standard
protein and low-protein diet groups were 250 g and 220 g, respectively, at the start of the experiment.

Each group was divided again into two groups according to the parameters to be observed; one group for measurement of liver function by means of red cell sequestration and the other for measurement of erythrocyte survival. Thus, the rats were finally divided into eight experimental groups (Table 1).

Table 1. Experimental schedules and grouping of rats.

| Group of rats            | Parameter measured | Time of measurement                              |
|-------------------------|--------------------|--------------------------------------------------|
| Standard protein diet   |                    |                                                  |
| Sham-operated           | Red cell survival  | 4 and 8 weeks after sham-operation               |
|                         | Liver function     | 6, 9 and 11 weeks after sham-operation           |
| Splenectomized          | Red cell survival  | 4, 6, 7 and 10 weeks after splenectomy           |
|                         | Liver function     | 2, 4, 5, 6, 8, 12 and 13 weeks after splenectomy |
| Low-protein diet        |                    |                                                  |
| Sham-operated           | Red cell survival  | 4 and 6 weeks after sham-operation               |
|                         | Liver function     | 3, 4, 6 and 9 weeks after sham-operation         |
| Splenectomized          | Red cell survival  | 5 and 8 weeks after splenectomy                  |
|                         | Liver function     | 3, 4, 5, 6, 8 and 9 weeks after splenectomy      |

Erythrocyte survival time \((T\ 1/2\ Rbc)\). Measurement of erythrocyte survival time was initiated at appropriate intervals starting four weeks after the operation to investigate the influence of time after splenectomy on the survival time. The procedures were as follows: Isologous ACD blood (collected in acid citrate dextrose solution) was taken by heart puncture from a donor rat of each dietary group, and labeled with 20–25 \(\mu\)Ci of radioactive sodium chromate ( Rachromate, Dainabot, Tokyo, specific activity; 42.3 mCi/mg \(^{51}\)Cr) per ml of blood. The blood was incubated with \(^{51}\)Cr at room temperature for 30 min. Incubation was terminated by adding 10 mg of sodium ascorbate per ml of blood. 0.5 ml of the labeled blood cells was injected into a tail vein of the experimental rat. Blood samples were taken from experimental 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 in tubes containing 1 ml of water. Radioactivity was counted by a well-type scintillation counter (Autogamma Spectrometer, Packard). Radioactivity measured 24 hr after injection was taken as 100% and that of samples counted on a particular day were expressed as percentage of the value of the 24-hr sample. Corrections were made for the hematocrit and the \(T\ 1/2\ Rbc\) was calculated on the decay curve of the radioactivity thus counted.

Osmotic fragility. Osmotic fragility of the red cells was measured in all experimental groups by a modified DACIE and VAUGHAM method. A series of hemolysis tubes was set up containing 10 ml of neutral saline of graded concentration 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 hemato-
crit value of approximately 33%. A 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 a wavelength of 545 nm. Hemolysis in the tubes was expressed as a percentage of that of a sample hemolyzed in water. The concentration of NaCl giving 50% hemolysis was intrapolated and the value obtained is termed here as the half hemolysis rate (HHR).

Sequestration rate of red cells. The sequestering function of liver and spleen was measured as follows: A rat from each group was chosen as a donor, and an appropriate volume of blood was taken by heart puncture in acid citrate dextrose (ACD) solution and red cells were labeled with 4 $\mu$Ci of radioactive chromate (Rachromate, specific activity; 42.3 mCi/mg $^{51}$Cr) per ml of the blood by incubation for 30 min at room temperature. The labeled cells, after being washed once, were brought to a 50% cell suspension in buffered isotonic saline (pH 7.4). This cell suspension was incubated with an equal volume of freshly prepared 12 mM N-ethyl-maleimide (NEM) solution for 1 hr at room temperature. This concentration of NEM solution is enough to form the stable addition products with sulfhydral compounds in the red cells (2). The NEM-treated red cells become susceptible to splenic uptake without altering their osmotic fragility. After the incubation, red cells were washed twice and resuspended in 0.9% saline at 50% concentration. 0.5 ml of thus treated red cells was injected into a tail vein of the experimental rat. Three hours after the injection, the animals were sacrificed by withdrawing blood via heart puncture under ether anesthesia, and immediately liver and spleen were weighed. A weighed portion of these organs and 1 ml of blood each were placed in the tubes and dissolved in 2 ml of 10% NaOH solution. Radioactivity was determined with a well-type scintillation counter. The total radioactivities of the whole organs and the total circulating blood were calculated by multiplying the sample counts by the weight of the organ or the blood volume. Blood volume was estimated by hematocrit value and plasma volume, which was measured by the dye dilution method using T-1824 (9). Correction of the blood volume was made by $F_{cells}$ value of rats (10). The sequestration rates in liver and spleen and the remaining rate of the labeled red cells in the circulation were expressed as percentage of the radioactivities injected.

Measurements of the standard protein diet groups were initiated starting two weeks after the operation. In the low-protein diet groups, measurements began three weeks after the operation. Four to six rats were sacrificed in a group at appropriate intervals after the operation.

RESULTS

Over 90% of the injected labeled red cells was recovered in liver, spleen and circulating blood. The rest of the cells was distributed in other organs and tissues.
not listed here. The overall average recovery of the injected radioactivity was 100%.

1) Standard protein diet group

The results of percentage distribution of labeled red cells in liver, spleen and circulating blood are summarized in Table 2.

| Condition                      | Number of rat | Liver (%) | Spleen (%) | Circulating blood (%) |
|--------------------------------|---------------|-----------|------------|-----------------------|
| Intact red cells               |               |           |            |                       |
| Sham-operated                  | 6             | 3.3       | 1.0        | 87.9                  |
|                                |               | ±0.9      | ±0.5       | ±2.1                  |
| NEM-treated red cells          |               |           |            |                       |
| Sham-operated                  | 7             | 10.0      | 32.8       | 51.0                  |
|                                |               | ±2.0      | ±5.8       | ±5.4                  |
| Splenectomized (4 weeks)       | 4             | 10.9      | 0          | 80.1                  |
|                                |               | ±1.7      |            | ±2.2                  |
| Splenectomized (8 weeks)       | 4             | 79.1      | 0          | 11.8                  |
|                                |               | ±6.8      |            | ±4.3                  |

Distributions of 51Cr-red cells are expressed as percentage recovery of total 51Cr injected. For details see text. Values are means ± SD.

In the sham-operated rats, approximately 88% of the intact red cells (cells not treated with NEM) remained in the circulating blood, and sequestration rates in liver and spleen were considerably low. The remaining rate of NEM-treated red cells in the circulation was reduced to 50%, and the sequestration rates of the cells in spleen and liver were increased to 33% and 10%, respectively.

On the splenectomized rats, the sequestration rate of NEM-treated red cells in liver was identical up to four weeks after the operation to that of sham-operated rats and 80% of labeled cells remained in the circulation, which was an equal amount of the sum of the distribution rates in spleen and the circulating blood of the sham-operated rats.

Eight weeks after splenectomy, the sequestration rate of NEM-treated red cells in liver reached 80%, and remaining rate in the circulation was reduced to 12%.

The changes in red cell sequestration rate in liver were examined in connection with the time after splenectomy and the results are shown in Fig. 1. Within four weeks after splenectomy, the sequestration rate of liver was identical to that of sham-operated rats. The sequestration rate of liver was gradually increased from the fifth week, and reached a maximum level at the eighth week. After this the sequestration rate declined, but still it remained at a higher level than that of sham-operated rats at the thirteenth week after splenectomy.

Osmotic fragility and survival time of red cells were measured after splenec-
Fig. 1. Changes in sequestration rate of NEM-treated red cells in liver after splenectomy. Each point and vertical bar represent the mean value ± S.D.

Fig. 2. Changes in osmotic fragility and erythrocyte survival after splenectomy in the rats fed a standard protein diet. Shaded areas represent the mean values ± S.D. of sham-operated rats. Each point and vertical bar represent the mean value ± S.D. Since there are no statistical differences among the values of splenectomized rats, average values after splenectomy are illustrated in the figure. Osmotic fragility of red cell is expressed as HHR which is the concentration of NaCl to give 50% hemolysis. For details, see text.

tomy in conjunction with the changes in hepatic sequestration rate of NEM-treated red cells. Figure 2 shows the changes in osmotic fragility and survival time of red cells. Osmotic fragility of red cells decreased after splenectomy and its diminished level was kept constant throughout the experimental period. This means that the osmotic fragility of red cells is independent to the changes in hepatic sequestration of the NEM-treated cells after splenectomy. The average survival time of red cells of the rats with intact spleen was 19.9 ± 1.8 days (T 1/2 Rbc).
The fluctuation of T1/2 Rbc with the time course after splenectomy was very slight. The overall average survival time of red cells of splenectomized rats was 21.9±1.5 days, which was significantly longer than that of the rats with intact spleens (P<0.001).

2) Low-protein diet group

Table 3 shows the percentage distribution of injected red cells in liver, spleen and circulating blood of rats fed a low-protein diet.

Table 3. Percentage distribution of 51Cr-red cells in liver, spleen and circulating blood of rats fed a low-protein diet.

| Condition                  | Number of rat | Liver (%) | Spleen (%) | Circulating blood (%) |
|----------------------------|---------------|-----------|------------|-----------------------|
| Intact red cells           |               |           |            |                       |
| Sham-operated              | 3             | 1.8       | 0.4        | 85.1                  |
|                           |               | ±0.2      | ±0.1       | ±2.3                  |
| Splenectomized             | 5             | 2.2       |            | 85.0                  |
|                           |               | ±0.4      |            | ±3.4                  |
| NEM-treated red cells      |               |           |            |                       |
| Sham-operated              | 5             | 7.6       | 20.8       | 64.5                  |
|                           |               | ±1.5      | ±1.1       | ±3.3                  |
| Splenectomized             | 12            | 6.9       |            | 84.3                  |
|                           |               | ±2.9      |            | ±4.2                  |

Distributions of 51Cr-red cells are expressed as percentage recovery of total 51Cr injected. For details see text. Values are means ± S.D.

85% of the intact red cells (cells not treated with NEM) remained in the circulation of both sham-operated and splenectomized rats.

The difference of sequestration rate in liver between splenectomized and sham-operated rats was 0.4%, which was the amount that the spleen should have sequestered. In the sham-operated rats, the respective sequestration rates of NEM-treated red cells in liver and spleen were 7.6% and 21%, and 64.5% of the cells remaining in the circulation. In the splenectomized rats, 84.3% of the NEM-treated red cells remained in the circulation, and only 6.9% was sequestered in liver. There observed no appreciable changes in sequestration rate of NEM-treated red cell in liver with the time course after the splenectomy as observed in the rats of standard protein diet.

Figure 3 shows the changes in osmotic fragility and survival time of red cells after splenectomy. Osmotic fragility was significantly decreased by splenectomy. There was a tendency toward increase in osmotic fragility with the time course after splenectomy.

18.5±2.0 days of T1/2 Rbc in splenectomized rats was significantly longer than that of 13.7±2.9 days in the rats with intact spleens (P<0.005). The survival time of red cells after splenectomy was almost constant throughout the experimental period.
Fig. 3. Changes in osmotic fragility and erythrocyte survival after splenectomy in rats fed a low-protein diet. Shaded areas represent the mean values ± S.D. of sham-operated rats. Each point and vertical bar represent the mean value ± S.D. Since there are no statistical differences among the values of splenectomized rats, average values after splenectomy are illustrated in the figure. Osmotic fragility of red cells is expressed as HHR which is the concentration of NaCl to give 50% hemolysis. For details, see text.

Table 4. Body and organ weights and percentage sequestration of NEM-treated red cells in liver and spleen per gram of wet organ.

| Diet and condition | Body weight (g) | Liver weight (g) | Weight (g/100g) | Sequestration rate (%) | Spleen weight (g) | Weight (g/100g) | Sequestration rate (%) |
|--------------------|-----------------|-----------------|-----------------|------------------------|-------------------|-----------------|------------------------|
| Standard protein diet |                 |                 |                 |                        |                   |                 |                        |
| Sham-operated (6–8 weeks) | 412 ± 27 | 10.35 ± 1.11 | 2.53 ± 0.13 | 1.01 ± 0.20 | 0.66 ± 0.09 | 0.16 ± 0.02 | 48.34 ± 5.59 |
| Splenectomized (4 weeks) | 312 ± 17 | 7.74 ± 0.59 | 2.51 ± 0.13 | 1.47 ± 0.39 | — | — | — |
| Splenectomized (8 weeks) | 404 ± 27 | 9.85 ± 0.80 | 2.45 ± 0.30 | 7.16* | — | — | — |
| Low-protein diet |                 |                 |                 |                        |                   |                 |                        |
| Sham-operated (3–9 weeks) | 263 ± 15 | 6.16 ± 0.63 | 2.37 ± 0.17 | 1.13 ± 0.27 | 0.37 ± 0.06 | 0.15 ± 0.03 | 48.13 ± 5.14 |
| Splenectomized (4 weeks) | 258 ± 16 | 6.63 ± 0.64 | 2.57 ± 0.18 | 1.54 | — | — | — |
| Splenectomized (8 weeks) | 274 ± 22 | 6.24 ± 0.52 | 2.28 ± 0.17 | 0.99 | — | — | — |

Sequestration rate (%) was calculated by dividing the total sequestration of organ by the weight of wet organ. Values are means ± S.D., and *means the value is statistically different (P<0.001) from that of sham-operated group.
The weights of liver and spleen were different among the two dietary groups. Accordingly, sequestration rates of NEM-treated red cells in liver and spleen were expressed per unit weight of organ, and the results are summarized in Table 4. The weight of spleen and the splenic sequestration were the same in both groups when they were expressed on the basis of unit body weight. The liver weight per unit body weight was also the same in all groups. The sequestration rate in the liver was the same in sham-operated rats regardless of the diet, and so was the rate in splenectomized rats within four weeks after splenectomy. At the eighth week after splenectomy, hepatic sequestration of standard protein diet group increased by five-fold in comparison with the value of the fourth week after splenectomy. But in the low-protein diet group, it was constant under all the experimental conditions.

**DISCUSSION**

Concerning the interrelationship of the reticuloendothelial activities among different organs, Jacob et al. (2) demonstrated that spleen, liver and bone marrow share the sequestration of red cells in such a way that the activity of liver and bone marrow is stimulated by the extra work load imposed on them after splenectomy. In the present experiment, red cell survival time was measured during periods when the compensatory response of liver after splenectomy was within the normal limit and at maximum. The red cell survival time, which started at a time of no compensatory response in liver, was $22.9 \pm 2.6$ days, and the survival time, which was measured at the time of approximate maximum compensatory response in liver, was $21.5 \pm 0.7$ days. There was no significant difference between the two values. Jacob et al. (2) suggested that the development of compensatory response of other reticuloendothelial functions might explain the inappreciable effect of splenectomy on red cell survival in otherwise normal individuals and might also, in part, account for late relapses following splenectomy in various hemolytic anemias. Our present experiment agreed with their report that the liver of splenectomized rats compensated the ability of sequestration of mildly damaged red cells, but it did not support their idea that the increased compensatory response of the liver might influence the survival time of red cells.

Since red cells become effete at the end of a normal life cycle (11), these effete and aged red cells would stimulate the proliferation of Kupffer cells in splenectomized rats. The compensatory responses of hepatic reticuloendothelial cells after splenectomy seemed to increase gradually, and to be imperfect in this situation, possibly because of an inability to duplicate certain anatomic features peculiar to the spleen.

Since the stimulation of the liver proliferation by the effete red cells is within physiological limits, no marked reticuloendothelial hyperplasia ensues the pathologic sequelae, and will not result in reducing the survival time and osmotic resistance of normal red cells.
Cellular proliferation in liver induced by effete red cells may be temporary and essentially reparative in nature, and this may be a reason for the declining tendency of compensatory response to the normal level after certain period of splenectomy.

In agreement with a previous report (4), we found that protein intake from diets had considerable effect on the survival time and osmotic fragility of red cells. 33% of NEM-treated red cells was sequestered by spleen in the rats with the standard protein diet, but the sequestration rate decreased to 20% in the rats with the low-protein diet. The sequestration rate of spleen per unit weight of the organ was identical (48%) in both dietary groups, therefore a low sequestration rate in spleen of the rats with the low-protein diet was attributed to a decreased weight of the spleen not to diminished activity per unit weight of spleen.

The osmotic fragility of circulating red cells decreased after splenectomy in agreement with the other reports (4–6, 12).

Hepatic compensatory sequestration in the rats with the low-protein diet was less than one-tenth of the maximum compensatory sequestration in the rats with the standard protein diet. 84% of NEM-treated cells injected to the rats of low-protein diet group remained unsequestered in circulation, whereas 12% of NEM-treated red cells remained in circulation in rats of standard protein diet group at the maximum compensatory response of liver.

The hepatic response to sequester the NEM-treated red cells may be related to the phagocytic activity of the Kupffer cells. DEO et al. (7) reported that depression of phagocytic activity in reticuloendothelial system (RES) was a consistent feature of protein-deficient monkeys and rats. The mechanism of depression of the phagocytic activity in RES is not properly understood. The influence of serum factors (opsonins) on the phagocytic activity of RES in protein-deficiency should be taken into account, while, DEO et al. (7) and RATNAKAR et al. (13) have excluded these humoral factors from responsible factors to depress the phagocytic activity by the experiments with protein-deficient monkeys and rats.

The hepatic blood-flow is known to modify the phagocytic activity of RES (14, 15). The role of hemodynamic factors cannot be assessed in the present study, however, we speculate that a decreased hepatic blood-flow in splenectomized rats on low-protein diets may not effectively increase the hepatic sequestration rate of NEM-treated red cells.

Thus the mechanism of depressed hepatic compensatory sequestration in protein deficient rats is a problem for future study.

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