Presence of Toxic Substances Which Inhibit Erythropoiesis in Serum of Uremic Nephrectomized Rabbits

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The presence of toxic substances which inhibit erythropoiesis in uremic sera is still undetermined. We examined the effect of aceton-methylalcohol extracts of serum of nephrectomized rabbits with uremia on the erythropoiesis.

The percentage red cell iron utilization was markedly reduced in nephrectomized rabbits with uremia. The administration of aceton-methylalcohol extracts of uremic rabbit serum resulted in a marked decrease of percentage red cell iron utilization in rats which fasted for 32 hours. However, the administration of aceton-methylalcohol extracts of normal rabbit serum did not result in any decrease of percentage red cell iron utilization in fasting rats. The administration of uremic extracts caused a marked shortening of the reticulocyte life span in colchicine rats. The percentage of erythroblasts labeled with H3-thymidine in vitro was markedly reduced in uremic nephrectomized rabbits. The administration of uremic extracts resulted in a decrease of the in vivo incorporation of H3-thymidine in erythroblasts with their increased abortion in rats which starved for 32 hours.

These results demonstrate the presence of toxic substances which inhibit erythropoiesis in uremic rabbit serum.

There has been reported an increasing body of evidence suggesting an etiologic role of the erythropoietin in the mechanisms of anemia associated with chronic renal failure. On the other hand, there are some evidences suggesting a significant role of toxic substances in uremic serum in the mechanism of the renal anemia. Dietrich and Sartorius reported that the degree of anemia in patients with chronic renal failure improved markedly, when the serum non-protein nitrogen level was reduced by hemodialysis. It is well established that there is a remarkable correlation between the degree of anemia and the serum non-protein nitrogen level in patients with chronic renal failure. These facts strongly suggest a pathogenetic role of toxic substances in uremic serum in the mechanism of anemia associated with azotemia.

However, the effect of toxic substances in uremic serum on the erythropoiesis still remains to be determined. Erslev, Mirand et al., and Gallagher et al. reported the presence of erythropoietic factors in serum of nephrectomized rabbits.
animals with the high non-protein nitrogen level. Some investigators found a slight increase of erythropoietin in approximately one-half of the patients with chronic renal disease. Therefore, the extracts of uremic serum which will contain no erythropoietic factors should be examined for the study of the inhibitory effect of toxic substances in uremic serum on the erythropoiesis. Thus the effect of aceton-methylalcohol extracts of uremic serum on the generation cycle of erythroblasts was investigated.

METHOD

1. Experimental animals
Rabbits weighing 2-2.5 kg and albino rats were used as experimental animals.

2. Preparation of aceton-methylalcohol extracts of uremic serum
Rabbits were nephrectomized bilaterally and scarified to obtain the uremic serum 5 days after the nephrectomy. The serum non-protein nitrogen level was more than 450 mg/dl. The aceton-methylalcohol extracts were prepared from the above uremic serum by the method described by Heimeyer. One volume of uremic serum was added to one volume of aceton-methylalcohol mixture (aceton: methylalcohol = 2:6). After gentle and thorough shaking the above mixture was centrifuged and the supernatant was collected. Aceton and methylalcohol were evaporated by heating the supernatant in the water bath and residues were resolved in the minimum volume of physiologic saline.

3. Percentage red cell iron utilization (%RCU)
The percentage red cell iron utilization following injections of Fe was determined in nephrectomized rabbits at various intervals after the nephrectomy.
Rats fasting for 32 hours received intravenous injections of Fe and the % RCU was determined 16 hours after the administration of Fe. The effect of uremic serum, its aceton-methylalcohol extracts and aceton-methylalcohol extracts of normal rabbit serum, which showed a similar non-protein nitrogen level with uremic extracts, on the % RCU was determined in the above fasting rats. These rats received four subcutaneous injections of 2 ml of uremic serum or above extracts at five-hour intervals.

4. The reticulocyte response following the bleeding
Aceton-methylalcohol extracts of uremic serum were injected subcutaneously into rats receiving subcutaneous injection of 0.2 mg/100 g of colchicine. And then 2 ml of blood were bled from the jugular vein of the above rats. Changes of peripheral reticulocytes following the bleeding were examined at an one-day interval for 5 days.

5. The in vitro incorporation of H-thymidine in erythroblasts
The in vitro incorporation of H-thymidine in erythroblasts was determined
in nephrectomized rabbits with uremia by the radioautography.

Labeling technic: 4.5 ml of the bone marrow cell suspension containing approximately $10^5$ cells per mm$^3$ was added to 0.5 ml of the stock solution containing H$^3$-thymidine. The final concentration of H$^3$-thymidine was $0.5 \mu$Ci per ml. The above mixture was incubated for one hour at 37°C, at which time smears were made on glass slides from the cell concentrates and fixed in methylalcohol. Smears were covered by films of autoradiographic emulsion. The films were developed after two-weeks’ exposure in a cold and dark room, and the smears were stained through the film with the Giemsa stain. A total 100 cells were enumerated at each degree of maturation and the percentage of labeled cells was determined.

6. The in vivo incorporation of H$^3$-thymidine in erythroblasts in rats receiving aceton-methylalcohol extracts of uremic serum

Rats, which had fasted for 24 hours and received four subcutaneous injections of 2 ml of aceton-methylalcohol extracts of uremic serum at a five-hour interval were given the intravenous injection of H$^3$-thymidine (400 $\mu$Ci per 100 g). At various intervals following the injection of H$^3$-thymidine, bone marrow puncture was performed and the percentage of labeled erythroblasts was determined on bone marrow smears.

RESULTS

1. % RCU in nephrectomized rabbits (Fig. 1)

The % RCU averaged 60% 2 days after injection of Fe$^{59}$ in five normal rabbits. It averaged 30% in five nephrectomized rabbits, when the radioiron was injected 30 minutes after the nephrectomy. It was markedly reduced, averaging 13.6% in five nephrectomized rabbits, when the radioiron was administered 4 days after the nephrectomy.

2. The effect of aceton-methylalcohol extracts on the % RCU in fasting rats (Fig. 2)

The % RCU determined 16 hours after injection of Fe$^{59}$ averaged 8.5% in 6 normal rats fasting for 32 hours. The administration of uremic serum into fasting rats resulted in no changes of % RCU. The administration of aceton-methylalcohol extracts of uremic serum resulted in a marked decrease of % RCU, averaging 4.8% in five fasting rats. However, the administration of aceton-methylalcohol extracts of normal rabbits serum did not result in any decrease of % RCU, averaging 8% in six fasting rats.

3. The effect of aceton-methylalcohol extracts of uremic serum on the reticulocyte response following the bleeding in colchicinized rats (Fig. 3)

In rats which were given the subcutaneous injection of colchicine, reticulocytes decreased within 2 days following the bleeding and then started to
Fig. 1. % RCU in nephrectomized rabbits.
1. control. 2. injection of Fe$^{+9}$ 30 minutes after nephrectomy. 3. injection of Fe$^{+9}$ 4 days after nephrectomy.

Fig. 2. The effect of aceton-methylalcohol extract on the % RCU in the fasting rats.

Fig. 3. Effect of aceton-methylalcohol extracts of uremic serum on the reticurocyte response.
(1) Colchicinized bled rats with no treatment.
(2) Colchicinized bled rats with injections of extracts.
increase, reaching the peak 5 days after the bleeding. The half time of reticulocytes life span was calculated from the initial decay curve of reticulocytes following the bleeding in colchicinized rats. It averaged 27 hours in control normal rats.

Aceton-methylalcohol extracts of uremic serum were injected into colchicinized rats and then they were bled. The decrease of reticulocytes following the bleeding was accelerated and the rate of the reticulocytes increase was markedly reduced in these rats. The half time of reticulocyte life span was remarkably shortened, averaging 14 hours.

4. The in vitro incorporation of H₃-thymidine in erythroblasts of nephrectomized rabbits with uremia (Fig. 4)

The bone marrow suspension from the nephrectomized rabbits with uremia was incubated with H₃-thymidine in vitro for 60 minutes at 37°C and then smears from the above suspension were examined for the in vitro incorporation of H₃-thymidine in erythroblasts.

The percentage of labeled basophilic and polychromatic erythroblasts averaged 60% and 33% in five normal rabbits, respectively. The percentage of labeled basophilic and polychromatic erythroblasts markedly decreased, averaging 19% and 9% in five uremic rabbits, respectively.

Fig. 4. The in vitro incorporation of H₃-thymidine in erythroblasts of nephrectomized rabbits with uremia.
5. The effect of aceton-methylalcohol extracts on the in vivo incorporation of \( H^3 \)-thymidine in normal rat erythroblasts (Fig. 5)

![Graph showing the percentage of labeled cells in the basophilic, polychromatic, and orthochromatic erythroblast groups as a function of time after \( H^3 \)-thymidine injections.]

Notes:
- Basophilic erythroblasts
- Polychromatic erythroblasts
- Orthochromatic erythroblasts

The top of Fig. 5 shows the curve of the average percentage of labeled erythroblasts determined in five normal rats as a function of time after the intravenous injection of \( H^3 \)-thymidine. The percentage of labeled basophilic erythroblasts averaged 70.3% one hour after injection of \( H^3 \)-thymidine. It reached the peak 9 hours after injection, averaging 86.5%, and then declined gradually. The percentage of labeled polychromatic erythroblasts averaged 26.7% one hour after injection of \( H^3 \)-thymidine. Its peak was present 12 hours after injection, averaging 80.0%. The labeled orthochromatic erythroblasts appeared three hours after injection of \( H^3 \)-thymidine and the percentage of labeled cells reached the peak between 12 and 24 hours after injection. In five rats which fasted for 24 hours, the initial percentage of labeled basophilic erythroblasts averaged 48%.
The peak of the percentage of labeled basophilic erythroblasts was present 16 hours after the injection of H³-thymidine and averaged 83%. The average initial percentage of labeled polychromatic erythroblasts was 21% and the peak averaging 70% was present 16 hours after the injection. Labeled orthochromatic erythroblasts were found 6 hours after the H³-thymidine injection and the labeling index reached the peak 24 hours after the injection.

The bottom of Fig. 5 shows the curve of the percentage of labeled erythroblasts which was determined in five rats which had fasted for 24 hours and had been given the injection of aceton-methylalcohol extracts of uremic serum. The percentage of labeled basophilic erythroblasts one hour after injection of H³-thymidine was markedly reduced, averaging 42%, in five rats which had fasted for 24 hours and had been given the injection of aceton-methylalcohol extracts of uremic serum. Its peak was present 6 hours after injection, averaging 60%. The percentage of labeled polychromatic erythroblasts averaged 23% one hour after injection of H³-thymidine. The labeled orthochromatic erythroblasts was demonstrated, and their percentage averaged 6% 6 hours after injection and reached the peak averaging 4% 16 hours after injection of H³-thymidine.

DISCUSSION

Jacobson et al. demonstrated the absence of reticulocytes production and a markedly reduced percentage red cell iron turnover in nephrectomized animals and suggested that the absence of the erythropoietin production in the kidney was the main pathogenetic factor of renal anemia in man. Naets denied the role of azotemia associated with chronic renal failure and concluded that the decreased erythropoietin production in the kidney was the pathogenetic factor. However, it is well known that a good correlation between the degree of anemia and the serum non-protein nitrogen level is observed in patients with chronic renal failure. Dietrich and Sartorius reported that the decrease of serum non-protein nitrogen level due to hemodialysis resulted in the recovery of the reduced erythropoiesis in patients with anemia associated with chronic renal failure. Erslev showed that the iron incorporation of normal rabbit bone marrow suspended in uremic rabbit serum with Fe⁵⁹ was markedly reduced. However, the proliferative index of erythroblasts was not changed. These facts suggest that some toxic substances in uremic serum might play a significant role in the mechanism of the reduced erythropoiesis in patients with uremia. Therefore, the effect of toxic substances in uremic serum on the generation cycle of erythroblasts was studied.

Many authors demonstrated the presence of erythropoietic activities in uremic serum. It is necessary, therefore, that erythropoietic activities of serum which might counteract toxic substances in uremic serum should be eliminated in experiments on the effect of toxic uremic substances on the erythropoiesis. Thus
aceton-methylalcohol extracts of uremic serum were used as toxic substances in this study.

The % RCU was markedly reduced in nephrectomized rabbits. This decrease of % RCU was more remarkable in rabbits receiving Fe⁵⁹ 4 days after the nephrectomy than in those receiving Fe⁵⁹ 30 minutes after the nephrectomy. The administration of aceton-methylalcohol extracts of uremic serum markedly reduced the % RCU in fasting rats, while the administration of uremic serum did not result in such a decrease of the % RCU. The administration of aceton-methylalcohol extracts of normal rabbits serum which contained non-protein nitrogen at approximately the same level as in uremic extracts, resulted in no decrease of the % RCU in fasting rats. These results strongly suggest that some toxic substances which are present only in uremic serum inhibit the erythropoiesis.

When two ml of blood were bled from rats receiving colchicine, their reticulocytes decreased within two days after the bleeding and then increased, reaching the peak 5 days after the bleeding. When rats receiving colchicine and aceton-methylalcohol extracts of uremic serum were bled, their reticulocytes showed the initial decrease and then increased, reaching the peak 5 days after the bleeding. However, the rate of the initial decrease was markedly accelerated and the rate of the following increase was markedly reduced in rats receiving aceton-methylalcohol extracts. The half time of reticulocyte life span calculated from the initial decrease curve was 27 hours in normal colchicinized rats and was 14 hours in rats receiving aceton-methylalcohol extracts. These results indicate that aceton-methylalcohol extracts prolonged the generation cycle of erythroblasts and shortened the life span of reticulocytes.

The in vitro incorporation of H³-thymidine in basophilic and polychromatic erythroblasts markedly decreased in nephrectomized rabbits, the percentage of labeled cells averaging 19% and 9%, respectively. This result indicates a prolongation of generation time of basophilic and polychromatic erythroblasts in nephrectomized rabbits with uremia.

The administration of aceton-methylalcohol extracts of uremic serum resulted in a marked decrease of the percentage of labeled basophilic and polychromatic erythroblasts in rats one hour after injection of H³-thymidine. This decrease of the initial labeling index indicates a prolongation of generation time of erythroblasts due to the administration of toxic uremic extracts. When we compare the curve of labeling index in rats which received uremic extracts with that in starved control rats, we are able to find a remarkable difference between them. The peak of the percentage of labeled basophilic erythroblasts was lower in rats which had received extracts than in control starved rats and it declined rapidly, indicating the presence of an abortion of labeled basophilic erythroblasts. The percentage of labeled polychromatic erythroblasts of rats which received uremic extracts reached the peak earlier than that of control starved rats, and it declined
rapidly. The curve of the percentage of labeled orthochromatic erythroblasts was lower in rats which had received uremic extracts than that in control starved rats. These results indicate increased abortion of labeled polychromatic erythroblasts.

From the above results, it is evident that some toxic substances in uremic serum markedly reduce the red cell iron utilization, prolong the generation cycle of erythroblasts and increase their abortion rate.

References