

Effects of Circulating Red Cell Mass on Diet-Induced Atrial Thrombosis in Mice¹

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Atrial thrombosis is a common lesion in female Taconic Swiss mice fed a high-fat (28%), low-protein (8%), hypolipotropic diet for 10 wk or longer. After the third week of such feeding the mice studied here were injected with either erythropoietin, washed, packed red blood cells, lysed red blood cells, plasma or physiological saline.

In mice receiving injections of lysed red cells, plasma or saline, respectively 75, 54 and 82% of those surviving for 10 wk had developed atrial thrombosis. Hematocrits were 9.3% or below in these groups. Hematocrits were maintained at an average of 33.0% in the erythropoietin group and 32.4% in the transfused (packed erythrocytes) group. Only one of the erythropoietin injected animals and none of the transfused animals developed atrial thrombosis. The evidence indicates that the anemia induced by the experimental diet results from lack of erythropoietin production or activity and that the hypoxia of anemia plays a role in the development of atrial thrombosis.

INTRODUCTION

A high incidence of atrial thrombosis and a concurrent severe anemia occur in mice fed a high-fat, low-protein, hypolipotropic diet (1-4). As the anemia increases in severity there is a concomitant rise in the incidence of atrial thrombosis. When the hematocrit level was maintained at a near normal level by ip injections of washed, packed red blood cells atrial thrombosis was prevented in mice fed this atypical diet (4). Whether the antithrombogenic action of the transfused erythrocytes was due directly to their oxygen-transport capacity or to erythropoietic, fibrinolytic or other activities remains undetermined.

Erythropoietin is known to initiate differentiation of the hemopoietic stem cell into erythroblasts and may be involved in accelerating erythroblastic maturation and the synthesis of hemoglobin. The exact cellular site(s) of production and bio-

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chemical mechanisms responsible for the action of erythropoietic factors are for the most part unknown (5). Studies of the action of erythropoietin in certain clinical erythropoietic disorders has provided information on the clinical problems and pathogenesis associated with these diseases (5). The role of erythropoietin in the diet-induced anemia of this study has heretofore not been determined.

The present study considers several aspects of the relation of erythrocytes and anemia to this type of diet-induced atrial thrombosis. The effects on atrial thrombosis of ip injections of erythropoietin, lysed erythrocytes and plasma were studied in mice fed the thrombogenic diet. A major purpose was to identify participants in the antithrombogenic process or activity.

MATERIALS AND METHODS

Portions of materials and methods used were as reported previously (4). Young, adult, nulliparous mice of the Taconic Swiss (TS) stock with initial body weights of 24–30 g and free of spontaneous cardiovascular diseases were used (6). Mice receiving the experimental diet, ad lib., were housed in cages with a raised wire mesh floor used to limit coprophagia. Within the mouse cages food was available in metal containers constructed with partitions to restrict the mouse's contact with the diet to its mouth and snout (7). The composition of the semipurified experimental diet was 28% fat (lard), 8% protein (casein), and 57% sugar (sucrose) supplemented with adequate amounts of vitamins and minerals and trace elements except choline (2, 8–9).

Mice were divided into five groups: group 1 (ten mice) was fed the experimental diet for 10 wk and in addition was injected daily beginning at the third week with 1.5 IU of erythropoietin (Step 1, Standard B-Connaught Medical Research Laboratories, University of Toronto) in 0.05 ml saline to maintain the hematocrit at approximately 30; group 2 (eight mice) received the experimental diet for 10 wk and in addition was injected weekly beginning at the third week with 0.25–0.5 ml of washed packed red cells as needed to maintain the hematocrit at approximately 30; group 3 (20 mice) received the experimental diet for 10 wk and in addition was injected daily (0.05 ml) or weekly (0.5 ml) beginning at the third week with lysed red cells; group 4 (19 mice) received the experimental diet for 10 wk and in addition was injected daily (0.05 ml) or weekly (0.5 ml) beginning at the third week with plasma; and group 5 (19 mice) received the experimental diet for 10 wk and in addition was injected daily (0.05 ml) or weekly (0.5 ml) beginning at the third week with saline. Animals were weighed and hematocrits determined weekly except for certain animals in the saline group (5) which developed closed, swollen or severely scarred eyes early in the experiment. Incidence of atrial thrombosis in non-injected mice (Table 1) fed the diet used here has been reported previously and included all animals which survived approximately 10 wk (4). Only those animals surviving for the full duration of this experiment (10 wk) were used for statistical purposes in Fig. 1 and Table 1.

Preparation of erythrocytes for injection has been described previously (4). Heparinized blood (7–8 ml) from the postorbital sinus of donor TS mice was centrifuged for 10 min at 2000 rpm and the plasma removed. The buffy coat was removed and the erythrocytes suspended in a quantity of saline sufficient to make 15 ml. Washing of the erythrocytes was repeated three times. The washed, packed, white blood cell (WBC)-free erythrocytes were injected ip into recipients.

Plasma removed from whole blood of donor mice that had not previously been

bled was used. Washed, packed, WBC-free erythrocytes collected in the same manner as those used for injection were frozen and thawed three times and then microscopically examined for extent of lysis. Only occasional macrocytic erythrocytes resisted this method of lysis.

Hearts were bisected at autopsy and after embedding in paraffin were sectioned at 8 μm in a frontal plane with care to preserve the shape and position of the atria. The PAS (periodic acid-Schiff's reagent) method plus hematoxylin and with diastase hydrolysis was used to demonstrate atrial thrombosis and as a routine survey stain. Connective tissue stains included the silver method of Gordon and Sweets, a combined PAS-silver method of Wilson *et al.*, and the Mallory aniline blue-acid fuschin method (10, 11).

Blood smears for microscopic examination were made from blood obtained from the post-orbital sinus (12). Brecher's new methylene blue technique for reticulocytes and Wright's stain technique were used to demonstrate reticulocytes and erythrocyte characteristics, respectively.

Erythropoietin was obtained from Connaught Medical Research Laboratories, University of Toronto, Willowdale, Ontario, Canada. It is a Step 1 grade with a potency of approximately 0.2–9.5 units/mg dry weight using the International Reference Preparation (Standard B) as a standard (13).

RESULTS

The results are summarized in Fig. 1 and Table 1. In those groups of mice (3, 4, 5) that received either a single large dose (0.5 ml) or daily small doses (0.05 ml) of injected material, the method of injection did not alter the results and they are considered together.

Weight loss in animals that were fed the experimental diet for 10 wk plus daily or weekly injections of lysed red cells, plasma or saline was -24 , -29 and -27% , respectively. These data do not differ significantly from those of previous studies using the same stock of mice and the same diet but without injections (1, 4). Ani-

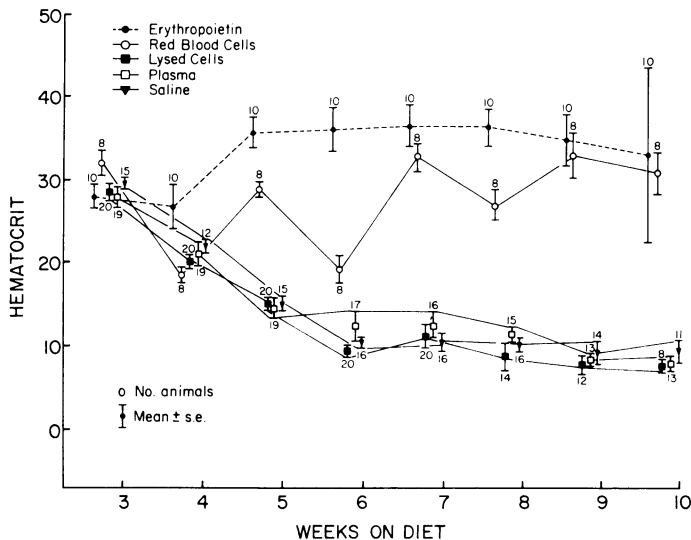


FIG. 1. Mean hematocrit values (weekly) in mice fed the experimental diet plus injections of erythropoietin, packed erythrocytes, lysed cells, plasma or saline.

TABLE 1
INCIDENCE (%) OF ATRIAL THROMBOSIS IN MICE FED THE EXPERIMENTAL
DIET PLUS INJECTIONS OF ERYTHROPOIETIN, PACKED ERYTHROCYTES,
LYSED CELLS, PLASMA OR SALINE

| Group | Treatment | Number of mice ^a | Incidence of atrial thrombosis (%) |
|-------|---------------------|-----------------------------|------------------------------------|
| 1 | Erythropoietin | 10 (10) | 10 ^b |
| 2 | Packed erythrocytes | 8 (8) | 0 ^b |
| 3 | Lysed cells | 20 (8) | 75 |
| 4 | Plasma | 19 (13) | 54 ^b |
| 5 | Saline | 19 (11) | 82 |

^a Number of mice in parentheses survived the duration of the experiment.

^b Significant difference ($P < 0.05$). Probability values were calculated with reference to an 84% incidence of atrial thrombosis in 25 non-injected mice fed the experimental diet for approximately 10 wk (4).

mals that received injections of erythropoietin lost only -7% body weight and those injected with erythrocytes had an increase of +6% in mean body weight.

Hematocrits of mice fed the experimental diet for 10 wk and given daily or weekly injections of lysed red cells, plasma or saline decreased rapidly and declined to a final mean level of 9.3% or less (Fig. 1). These data agree with those of previous studies using the same stock of mice and the same diet but without injections (4, 14). The final mean hematocrit of mice in the erythropoietin-injected mice was 33.0% and in the transfused group 32.4%. In the latter two groups, it was attempted to maintain hematocrits at approximately 30%, however, unlike the results of pilot studies, hematocrits of erythropoietin-injected mice leveled off at slightly above 35% by the fifth week and in transfused mice increased erratically from a fourth week level of 18.6% to the ninth week high of 36.0% (Fig. 1).

Only one of the ten erythropoietin-injected mice developed atrial thrombosis. Erythropoietin injections failed to maintain the hematocrit at the proper level in this animal. The hematocrit decreased to 22% after 4 wk and was 20% after 10 wk. The incidence of atrial thrombosis in groups 2-5 were 0, 75, 53 and 82%, respectively (Table 1). Statistically, plasma-injected mice may have been protected somewhat from atrial thrombosis compared to non-injected mice on the diet (Table 1), however no major increase in mean hematocrit values or other protective mechanisms was evident (Fig. 1). Histological changes during the formation of atrial thrombi have been described in detail previously and did not differ in this study (4).

As anemia increased in severity a rapid decrease of reticulocyte formation occurred in mice fed the experimental diet for 10 wk plus daily or weekly injections of lysed red cells, plasma or saline (groups 3-5). In addition to the decrease in reticulocytes the anemia was characterized by anisocytosis and hypochromia. Ball and Westin also found reticulocytopenia in mice fed the experimental diet only and demonstrated that the anemia was a microcytic and hypochromic type (3). Some anisocytosis and hypochromia occurred in the erythropoietin-injected mice, however, reticulocytosis occurred throughout the experimental period in response to the erythropoietin injections.

In mice that received erythropoietin injections the spleens were enlarged. Heme pigment was observed in most organs of mice injected with lysed red cells. A high

incidence (55%) of coronary thrombosis was observed in animals that received injections of lysed red cells. This was unexpected since in numerous previous studies using the same diet as fed here coronary thrombosis at the tenth week was not a significant finding (1-4). Except those mentioned above, changes in other organs of all the experimental groups were those expected in mice fed the diet used here.

DISCUSSION

The results demonstrate that it is the viable red cell and its prevention of hypoxia that is protective against thrombosis since neither plasma nor lysed cells increased the hematocrit or prevented atrial thrombosis. The anemia (microcytic, hypochromic) produced by feeding protein-deficient diets was readily reversed at any stage with return to proper nutrition (15, 16). Hematocrits returned to normal and atrial thrombosis was prevented in mice returned to a normal diet after being fed the diet used here for a period sufficient to induce anemia and a high incidence of atrial thrombosis (3).

In this study, lysed red cells were expected to have an erythropoietic effect (17-19). Brown *et al.* injected normal and fasted, dehydrated rats with washed lyophilized or freeze-thawed erythrocytes and obtained both a significant increase in ^{59}Fe incorporation and reticulocytosis (20). The erythropoietic stimulating activity was in the hemoglobin and heme-related compounds but not in the stroma or globin fractions of the red cells. Here the lysed cell homogenate did not prevent or correct the anemia of mice. Erythropoietic activity of red cell homogenates may, therefore, be a species characteristic (19).

A fibrinolytic or thrombolytic effect of lysed red cells has been reported (21-22). If thrombi develop by deposition of fibrin upon or under the endothelium (23-25) or at sites of "endothelial rupture" (4) the red cell fibrinolytic system could alter or prevent mural atrial thrombosis. Injection of lysed red cells of equal volume and concentration as the intact red cell injections were not effective in preventing atrial thrombosis. Thus, in this study hemolysis either destroyed the fibrinolytic actions of the red cells or the fibrinolytic process plays no role in the prevention of diet-induced atrial thrombosis (26).

Reissmann reported that protein deficiency anemia could be reversed by erythropoietin (16). Prior to this, it was shown that erythropoietin enhanced iron uptake and heme synthesis *in vitro* (27-28). Exogenous erythropoietin injection over a two wk period in preliminary studies by these authors (unpublished) reversed the diet-induced anemia at any stage of the dietary period and at any hematocrit from 6.7% upward. This provides an hypothesis that decreased erythropoietin production or activity was responsible for the anemic state which in turn predisposed the animals to extreme hypoxia and subsequent endocardial damage and atrial thrombosis. In this study erythropoietin-injected mice maintain a higher mean hematocrit level than erythrocyte injected mice; however, the range was greater and the one incidence of atrial thrombosis was from an animal that showed only a weak response to erythropoietin.

Microscopic examination of the blood of the erythropoietin-injected animals during the tenth week of the experiment demonstrated hypochromia of mature red cells and numerous reticulocytes. Normally an increase in hemoglobin occurs concurrently with red cell proliferation in erythropoietin-stimulated animals (29-30). However, with time hemoglobin synthesis ceases to be a response to erythropoietin

stimulation in animals severely deficient in essential nutrients (31). Erythropoietin-injected mice may be susceptible to atrial thrombosis not because of mechanical mechanisms (e.g., reduced circulating red cell mass) but because of a decrease in hemoglobin synthesis even though the hematocrit is near normal. Here massive hepatic liposis as a result or sequel of nutritional deficiencies may have reduced hemoglobin formation.

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