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Proline Induced Hemolytic Anemia in Fascioliasis (41916)

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Abstract. A secondary anemia is characteristic of infections with the liver fluke, *Fasciola hepatica,* and previous studies had suggested that proline released by the worm might be involved in producing this condition. In the current study the effect of fascioliasis on erythropoiesis was compared to the effects of proline infusion. Both treatments produced effects characteristic of rapid erythrocyte turnover due to hemolysis. Moreover, infusion of a proline analog into animals with *Fasciola* or into animals infused with proline prevented the anemia. To our knowledge the production of hemolytic anemia by excessive proline has not been previously reported.

A variety of human and veterinary diseases are complicated by an anemia secondary to the infection. Here we report that excessive levels of the amino acid proline can cause a secondary hemolytic anemia. Proline's effect is demonstrated in fascioliasis, a common and costly disease of sheep and cattle. This disease is caused by the bile duct fluke, *Fasciola hepatica,* that releases large quantities of proline into its hosts. Previous investigations had found that infused proline could mimic some symptoms of fascioliasis: Enlargement of the main bile duct (1) and an anemia (2). This report establishes that the anemia is hemolytic, and links the hemolytic state to proline coming from the worm by showing that a proline analog can reverse its anemic effect.

Materials and Methods. *Animals.* Male Wistar rats obtained from Royalhart Laboratories were infected by gavage (3, 4) with 25 *Fasciola* cysts using metacercariae from Baldwin Enterprises, Monmouth, Oregon. All rats were fed Agway Stress Diet 3000, and watered *ad libitum.* Animals were housed in an air conditioned facility (28–24°C) with lighting from 0700 to 2000 hr daily. Care and handling of animals were in accord with the *NIH Guide for the Care and Use of Laboratory Animals* (DHEW Pub. No. (NIH) 78-23).

Plasma volume study. In order to obtain data from rats with newly mature and older infections, 30 rats of approximately similar age and weight (100 ± 20 g) were divided into three groups: Eight animals were used as controls, eleven were infected immediately with *Fasciola,* and the remainder were infected 8 weeks later. Because mortality was anticipated in the infected groups, they contained more animals.

Sixteen weeks from the start of the experiment, when the infections were mature, plasma volume was measured in all groups by the radioiodinated serum albumin (RISA) technique (5, 6). Albumin (Sigma Chemical Co.) was iodinated with ^{125}I using a kit from New England Nuclear. After weights were obtained and the animals anesthetized with 7.0 ng/mg body wt sodium pentobarbital (Abbott Laboratories), bilateral incisions were made in the cephaloventral thorax to expose the jugular veins. Exactly 0.06 cc (1.8 nCi)/g body wt of RISA was injected into the left jugular vein of each rat. Fifteen minutes later, about 3 cc of blood was withdrawn from the right jugular vein and centrifuged for 4 min in a clinical centrifuge. One cubic centimeter of the plasma supernatant was drawn off and mixed with a fluor for counting in a Packard Tri-Carb liquid scintillation counter. Plasma volume was determined by dividing the radioactivity in the injection dose by that in the plasma sample.

Marrow morphology. Marrow samples were obtained from rats subjected to the following

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treatment. Twenty-four rats of similar age and weight (215 ± 15 g) were divided at random into three equal treatment groups. Each animal was intraperitoneally implanted with a 2 × 3-cm nylon-mesh sack and a PE-50 cannula. Sack material implantation of flukes and other surgical techniques were similar to those described elsewhere (1, 2).

All groups were infused with saline (0.85%) through the cannulas using the apparatus of Sawma *et al.* (7), but treatment of each group differed as follows: In the first group, five live *F. hepatica* obtained from donor rats were placed in the mesh sack of each animal. In the second group, the sacks were empty, but the saline infusate contained 20 m*M* L-proline. A third group served as a sham control, without worms or infused proline. In order to approximate conditions in mature infections, only adult flukes were implanted and the infusion rates of proline were adjusted so that animals received 85 μmole/animal per day (8).

After 2 weeks of infusion, each rat was anesthetized with ether and a 1-cc blood sample was taken by cardiac puncture. Blood smears were made by the coverglass technique and stained with May–Grunwald–Giemsa stain (9). Red cell counts and hematocrit (Ht) were determined immediately using previously described methods (2, 10). After killing, the right femur was excised from each rat. Then using a scissor, an epiphysis was removed, the femur was split longitudinally, and a bit of marrow was removed with a brush and mixed with hypotonic calf serum in a small beaker. Smears were immediately made from the mixture using the technique described above for blood smears. Differential counts of 500 cells were made from each slide. The results were independently verified by a second investigator with a 200-cell differential count; then both results were combined. Finally, myeloid to erythroid (M:E) ratios were calculated for each animal from the composite count.

Proline analog infusions. Rats were selected at random from among 25 of similar age and weight (150 ± 25 g), to make five equal treatment groups. Animals were prepared for infusion using the same techniques described above in the bone marrow study. Each rat was implanted with a mesh sack and a PE-50 cannula. All groups were infused with saline through the cannula but the treatments for each group differed as follows: In the *Fasciola* implanted group (Imp), five live adult *F. hepatica* were inserted into each sack. Animals in the proline infused group (PI) had no worms in the sack, but the saline infusate contained 20 m*M* L-proline. A third group (Imp/Dp) had five *Fasciola* in each sack and was infused with 20 m*M* 3,4-dehydroproline (3,4-DHP). The dehydroproline was a gift from Hoffman–LaRoche, Inc., Rahway, New Jersey. No worms were implanted in a fourth group (PI/Dp) where the infusate contained 20 m*M* proline and 20 m*M* 3,4-DHP. The fifth saline infused group served as a sham control (SC) and received no worms or test compound.

After 14 days of infusion, the animals were anesthetized with ether, and 2 cc of blood was withdrawn by cardiac puncture. Using the same methods as in the marrow study, RBCs per cubic millimeter and Ht were determined immediately. Samples were then frozen for total hemoglobin analysis, completed later using methods described elsewhere (10).

Statistical analysis. Differences between groups in the hematological parameters were evaluated using the Student Newman-Keuls (SNK) test and analysis of variance (ANOVA) procedures (11). Nonparametric Kruskall–Wallis and STP tests were used to measure differences in the M:E ratios from the bone marrow morphology study (12). To analyze the MCHC percentages and MCV values, an arcsine transformation was applied before the ANOVA–SNK (11). All error ranges are standard deviations.

Results. The results of the plasma volume study (in cc/kg body wt) indicated no significant differences between uninfected rats (31.2 ± 7.16), rats with 8-week infections (27.2 ± 4.27), and rats with 16-week infections (31.4 ± 2.81). Hence, the anemia was not due to hemodilution. Therefore, studies to determine the type of anemia were continued. Study of bone marrow morphology yielded the following M:E ratios: Median values were 1.6 for the *Fasciola* implanted group, 2.2 for the proline infused group, and 4.7 for the saline control group, respectively. Significant differences were found between the saline

control and both the proline infused and *Fasciola* implanted groups.

Although blood smears from saline infused animals appeared normal, smears from proline infused and *Fasciola* implanted animals showed polychromasia, macrocytosis, and anisocytosis. These observations, in conjunction with lower M:E ratios and high fecal urobilinogen levels, indicate a rapid erythrocyte turnover (RET) in the proline infused and *Fasciola* implanted animals.

Observations from a representative experiment examining the erythropoietic consequences of the interaction between proline and 3,4-DHP are shown in Table I. As indicated by millions of RBCs per cubic millimeter, MCV, and total hemoglobin (Hb) concentration, proline infusion and *Fasciola* implantation cause an apparent macrocytic anemia. It is important to note that in all parameters measured, the data show no significant difference between the anemic effects produced by proline infusion and those produced by *Fasciola* implantation. Of equal importance is the indication that 3,4-DHP mitigated the effects of infused proline and proline from the worm in RBCs, MCV, and total Hb concentration.

Fecal urobilinogen levels and MCHC show a trend toward inhibition of proline's effect by 3,4-DHP. However, a limited sample number and high variability in the data weaken the power of the statistical test, making determination of 3,4-DHP's effect on these parameters equivocal.

Discussion. The results of the current study demonstrate that the anemia produced by proline infusion is similar to that produced by *Fasciola* implantation, and they suggest that proline from the worms causes a hemolytic anemia. Furthermore, the inhibition of the anemia by 3,4-DHP in implanted and infused animals indicates that the anemia is due to proline from the worm.

Symptoms of RET have been reported in earlier studies on fascioliasis, including a

TABLE I. EFFECTS OF 3,4-DEHYDROPROLINE ON THE ANEMIA OF RATS INFUSED WITH PROLINE OR IMPLANTED WITH LIVE *Fasciola*

Group	RBC (10^6/mm^3)	MCV (μm)	Total Hb concentration (g/dl)	Fecal urobilinogen (mg/100 g)	MCHC (%)
			Data[a]		
Imp	5.82 ± 0.92	54.08 ± 9.97	9.82 ± 0.96	14.50 ± 2.70	31.6 ± 4.12
PI	6.32 ± 1.08	50.98 ± 7.13	10.10 ± 0.80	11.52 ± 3.71	31.7 ± 1.02
Imp/Dp	8.22 ± 0.61	41.28 ± 7.97	11.96 ± 0.61	10.52 ± 2.02	36.2 ± 7.21
PI/Dp	9.35 ± 0.29	39.91 ± 4.01	12.80 ± 0.29	10.41 ± 3.39	35.3 ± 2.97
SC	9.36 ± 0.76	37.03 ± 5.33	12.70 ± 0.58	6.75 ± 1.80	37.1 ± 3.35
			ANOVA analysis		
	$F(4, 21) = 31.63$ $P < 0.001$	$F(4, 21) = 11.49$ $P < 0.001$	$F(4, 21) = 29.53$ $P < 0.01$	$F(4, 21) = 4.37$ $P < 0.05$	$F(4, 21) = 1.93$ NS
			SNK analysis[b]		
Imp	*Imp/Dp, PI/Dp, SC*	*Imp/Dp, PI/Dp, SC*	*Imp/DP, PI/Dp, SC*	*SC*	—
PI	*Imp/Dp, PI/Dp, SC*	*Imp/Dp, PI/Dp, SC*	*Imp/Dp, PI/Dp, SC*	*SC*	—
Imp/Dp	*Imp, PI, PI/Dp, SC*	*Imp, PI*	*Imp, PI*		—
PI/Dp	*Imp, PI, Imp/Dp*	*Imp, PI*	*Imp, PI*		—
SC	*Imp, PI, Imp/Dp*	*Imp, PI*	*Imp, PI*	*Imp, PI*	—

[a] Standard hematological indices and fecal urobilinogen levels from a representative experiment. Data are means ± SD.

[b] Groups (in italics) listed under each parameter differ significantly ($P < 0.01$) from the group in the first column according to the SNK test. Abbreviations are: Imp, *Fasciola* implant; PI, proline infusion; Imp/Dp, *Fasciola* implant with 3,4-DHP infusion; PI/Dp, proline/3,4 DHP-cocktail infusion; SC, saline infused control.

decrease in M:E ratio with erythroid hyperplasia in marrow, (14, 15) and an accelerated erythropoietic iron turnover (13, 15, 16). In some of these investigations RET was attributed to biliary hemorrhaging and to hematophagia by the fluke (14–17), although hemolysis was not ruled out (14, 17). Previous studies in our laboratory, however, demonstrated that intraperitoneally infused proline (2) and implanted live *Fasciola* (10) produce an anemia similar to that of fascioliasis. Implantation eliminated possible hemorrhage due to hematophagia or mechanical injury by the worms. Because the present study demonstrates RET in the absence of hemorrhage or ineffective erythropoiesis, hemolysis is indicated. An effective erythropoietic response in host animals of *Fasciola* is shown by polychromasia and anisocytosis observed in this study and reculocytosis observed in earlier studies (10). Hemolytic anemia is also characterized by rapid erythrocyte destruction. Splenomegaly, an indicator of such destruction, has been reported in fascioliasis (18) and was observed by us in the course of other studies on fascioliasis (unpublished observations). Increased fecal urobilinogen levels (10) and observation of a decrease in erythrocyte lifespan during ^{51}Cr studies (17, 19) are also consistent with accelerated erythrocyte destruction. This conclusion does not rule out some biliary hemorrhaging and mechanical lysis of erythrocytes that may produce minor effects on the erythron. Although there was a trend toward lower MCHC, hypochromia, characteristic of fascioliasis (20, 21), was not observed in morphological examinations of blood smears from *Fasciola* implanted and proline infused animals. This absence of well-developed hypochromia is probably due to the short 2-week infusion period. Longer implantation studies without infusions have documented development of hypochromia (10). Therefore, a longer infusion period in future studies might induce hypochromia. Further studies are needed to determine the nature of the hemolysis. Possible extra vascular hemolysis due to increased splenic sequestration is indicated by splenomegaly. However, metabolic studies on erythrocytes by Phang and co-workers (22) reported that proline inhibits the stimulatory effect of pyrroline-5-carboxylate on the hexosemonophosphate pathway. Alteration of this or other important metabolic pathways may lead to intravascular hemolysis.

Excessive proline is also present in many other hepatic diseases associated with secondary anemias such as schistosomiasis (23) and chronic alcoholism (24). An understanding of the role of excessive proline in the pathophysiology of fascioliasis, as well as other major health and veterinary problems, could lead to the development of corrective therapy.

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