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Studies of the Hemopoietic Microenvironment. V. Erythropoietin-Induced Release of Vasoactive Substance(s) from Erythropoietin-Responsive Stem Cells[1] (39902)

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Significant reductions in blood flow occur in the spleens of polycythemic mice with suppressed erythropoiesis (1). Restoration of flow to levels seen in normal animals is achieved when erythropoietin (Ep) is administered (2) or when circulating Ep levels are elevated during hypoxic erythropoietic stimulation (3, 4). The vascular response induced by Ep is not immediate but is first seen 4–6 hr following the administration of Ep; it peaks at 24 hr, is markedly reduced by 72 hr, and is abolished by 96 hr after a single injection of Ep (2). This response is blocked by anti-Ep serum (3, 4). In addition, the limited splenic blood flow in W/W^v anemic mice which have deficient stem cells can be restored to normal following an infusion of normal stem cells (5). Based on these data, McCuskey *et al.* (1–5) have hypothesized that blood flow through the erythropoietic splenic red pulp is regulated locally by a vasoactive substance(s) which is released from some early erythroid cell, possibly the erythropoietin-responsive stem cell (ERC), in response to Ep.

The purpose of the present research was to determine more precisely the cellular origin of the vasoactive substance(s) and whether or not the vascular response induced by Ep was dependent upon stimulation of ERC by Ep. The experimental design was based on the following: (a) Myleran differentially suppresses uncommitted stem cells (CFU) more effectively than ERC and thus permits replication and differentiation of ERC in response to Ep without a significant inflow of CFU into ERC (6–8); (b) when a split dose of Ep is administered, 3 days apart, to polycythemic mice treated with Myleran, the first injection elicits replication of ERC while the second injection elicits differentiation of ERC into pronormoblasts (7); and (c) these responses to Ep can be blocked by hydroxyurea (7).

Accordingly, alterations in the splenic microvasculature that accompanied the suppression and recovery of CFU and ERC were examined in mice treated with Myleran. To determine more precisely which phase of ERC cellular activity (replication or differentiation) might be associated with Ep-induced changes in the splenic microvasculature, the spleens of polycythemic, Myleran-treated mice also were studied following the administration of Ep.

Materials and methods. Female CF_1 mice weighing approximately 20 g were fasted 2–4 hr and then fed Myleran (44 mg/kg body weight) through a gastric tube. The Myleran was dissolved in acetone (100 mg in 10 ml) and then diluted to 50 ml with corn oil immediately before feeding. Mice were studied 5, 10, 16, and 32 days post-treatment (see Table I). Normal, untreated mice and mice fed comparable amounts of the vehicle 5 days prior to observation served as controls.

Another group of female CF_1 mice weighing approximately 20 g was made polycythemic by intermittent exposure to a simulated altitude of 23,000 ft over 10 days for a total of approximately 210 hr. Only animals having a hematocrit in excess of 60% were used. Following the protocol of Reissman and Udupa (7), these mice were subdivided into five groups and treated as follows (see Table I): (a) Mice, 4 days posthypoxia, were fed Myleran as above and studied 2, 4, 6, 8, and 10 days postfeeding; polycythemic mice not fed Myleran served as controls and were observed at times equivalent to Days 0, 5, and 10; (b) similarly treated mice were given 8 U of Ep[2] sc 24 hr after administration of the Myleran and were studied 1, 2, 4, and 6 days after the injection of Ep; (c) mice treated as in (b) received a second injection

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[2] Erythropoietin (step III) was obtained from Connaught Laboratories.

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TABLE I. EXPERIMENTAL GROUPS OF MICE.

Treatment	Days[a] 0	1	2	3	4	5	6	7	8	9	10	16	32
Control, no treatment						X							
Control, corn oil and acetone (CA)	CA					X							
Myleran (M) dissolved in corn oil and acetone	M					X					X	X	X
Polycythemia, no treatment	X					X					X		
Polycythemia and Myleran (M)	M		X		X		X		X		X		
Polycythemia and Myleran (M) + single dose of Ep	M	8 U Ep	X	X		X		X					
Polycythemia and Myleran (M) + single dose of Ep and hydroxyurea (H)	M	8 U Ep + H	X										
Polycythemia and Myleran (M) + split dose of Ep	M	8 U Ep			4 U Ep	X	X	X					

[a] X indicates time of *in vivo* study and autopsy.

of 4 U of Ep sc 3 days after the first Ep injection and were studied 1, 2, and 3 days after the second injection of Ep; (d) mice treated with Myleran as in (a) were injected 24 hr later with 8 U of Ep sc and hydroxyurea (1.75 mg/kg body weight) ip followed 7 hr later by a second dose of hydroxyurea. An additional group of these mice was not given Ep and served as controls. Hydroxyurea was dissolved in distilled water so that approximately 0.3 to 0.4 ml was administered per injection. Mice were studied 24 hr after the Ep injection.

The spleens of at least five mice from each of the above groups were examined using the *in vivo* microscopic method described by McCuskey *et al.* (1). The relative amount of blood flow through the red pulp was determined by counting the number of channels in the red pulp that contained blood flow in a total of 10 microscopic fields (346, 185 μm^2) for each mouse (1).

Following *in vivo* microscopic examination, body weight, hematocrit, and 24-hr ^{59}Fe incorporations in the blood were determined. The latter was determined by injection of 0.5 μCi ^{59}Fe sc 24 hr prior to *in vivo* microscopic study and autopsy. The percentage of ^{59}Fe incorporation into red blood cells was calculated using 7.3% of body weight as an estimate of total red blood cell volume in the polycythemic mice (1, 2, 11); 6.2% was used as an estimate of total red blood cell volume in the normal mice (11).

Standard errors were calculated and levels of significance were determined using Fischer's *t* test. Using the latter, *P* values greater than 0.05 were considered to be significant.

Results. The results of the experiments using normal mice treated with Myleran are shown in Fig. 1. There were no significant differences in the incorporation of ^{59}Fe or in the number of channels in the splenic red pulp containing blood flow between the two control groups. Hematocrits (normal, 45 ± 1.32%; corn oil-fed, 48 ± 0.52%) also were not significantly different.

Five days after administration of Myleran, there was a reduction in the microcirculation, as shown by the decreased number of channels with flow. This was accompanied by a depression in erythropoiesis, as indicated by the marked reduction in ^{59}Fe incorporation. The hematocrit, however, was still relatively normal (43 ± 1.02%). By Day 10 the microcirculation returned to control levels, however, incorporation of ^{59}Fe, although greater than on Day 5, was still below the corn oil control ($P > 0.02$), as was the hematocrit (40 ± 1.68%) ($P > 0.01$). It should be noted, however, that compared to the nontreated control, the difference in ^{59}Fe incorporation was not statistically significant, but the difference in hematocrits was ($P > 0.05$). By Day 16, incorporation of ^{59}Fe had returned to control levels, but the hematocrit remained below normal (36 ± 3.02%). By Day 32 the hematocrit (46 ± 0.88%) and the ^{59}Fe incor-

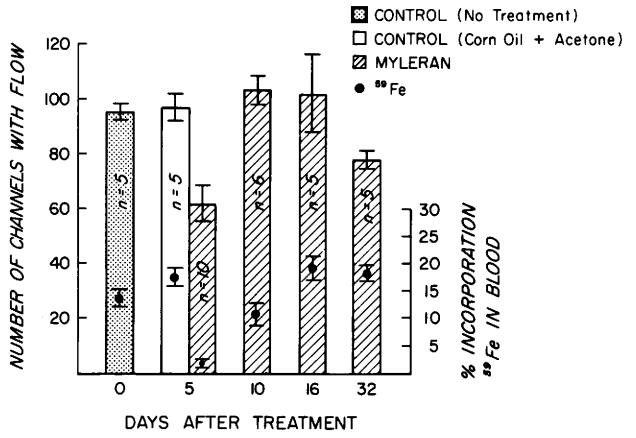

FIG. 1. Effects of Myleran on the mean number (±SE) of channels containing blood flow in 10 microscopic fields (346,185 μm^2) of the splenic red pulp of normal mice and on the mean (±SE) ^{59}Fe incorporation in the blood. *N*, number of animals.

poration had returned to control levels, but there was some reduction ($P < 0.02$) in blood flow through the red pulp.

In contrast, Fig. 2 shows that, in untreated polycythemic mice and those treated only with Myleran, the splenic microcirculation was below normal and remained low for at least 10 days after treatment. This was accompanied by a markedly depressed uptake of ^{59}Fe. All polycythemic animals had hematocrits in excess of 60%. Figure 3 (left side) illustrates that the administration of 8 U of Ep to such polycythemic mice treated with Myleran resulted in a significant increase in the microcirculation 24 hr later; this response was nearly abolished by 48 hr postinjection. No increase in the uptake of ^{59}Fe was noted at these times, but there was a significant increase on Day 4 which disappeared by Day 6.

The marked increase in microcirculation 24 hr after the administration of Ep to polycythemic mice treated with Myleran was prevented by hydroxyurea (Fig. 3, left side). Fig. 3 (left side) also shows that hydroxyurea alone did not alter the microcirculation or ^{59}Fe incorporation of polycythemic mice treated only with Myleran (Day 2, Fig. 2). When comparing Figs. 2 and 3, it should be noted that 1 day after the first injection of Ep is 2 days after the administration of Myleran (see Table I).

The right side of Fig. 3 shows that following a second dose of Ep (4 U), a limited increase in the microcirculation occurred 24 hr after the injection. However, this response was much smaller than that obtained following the initial injection of Ep, but was significantly above the baseline levels illustrated in Fig. 2. Two days later this second

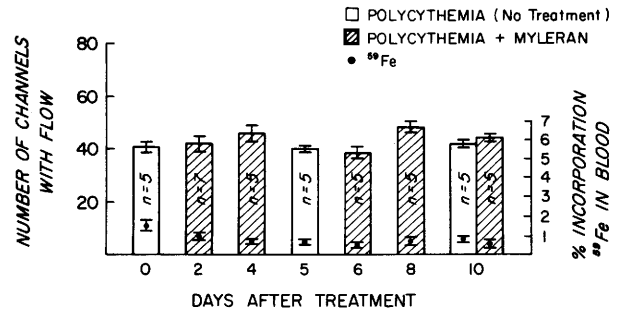

FIG. 2. Effects of Myleran on the mean number (±SE) of channels containing blood flow in 10 microscopic fields (346,185 μm^2) of the splenic red pulp of polycythemic mice and on the mean (±SE) ^{59}Fe incorporation in the blood. *N*, number of animals.

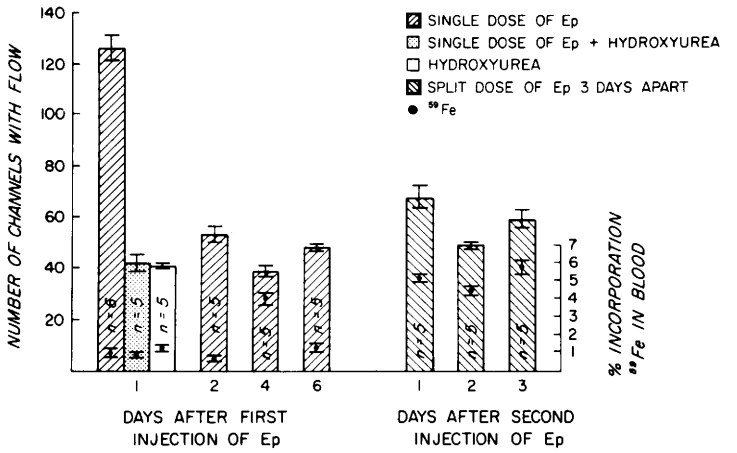

FIG. 3. Effects of a single dose of erythropoietin (8 U) alone and in conjunction with hydroxyurea and a split dose of erythropoietin (8 and 4 U) administered 3 days apart on the mean number (±SE) of channels containing blood flow in 10 microscopic fields (346,185 μm^2) of the splenic red pulp of polycythemic mice treated with Myleran and on the mean (±SE) ^{59}Fe incorporation in the blood. *N*, number of animals.

response approached baseline levels, although a slight response was seen on Day 3. Incorporations of ^{59}Fe were significantly elevated 24, 48, and 72 hr following the second injection of Ep.

Discussion. The results of this study substantiate the hypothesis of McCuskey *et al.* (1-5) that the effect of Ep on the microvasculature of the erythropoietic mouse spleen is not direct, but is mediated by some vasoactive substance(s) released from ERC following stimulation by Ep.

Five days after administration of Myleran to normal mice which have circulating Ep, there was a significant reduction in blood flow through the red pulp. This response coincided with the reported interval of time of suppression of ERC by Myleran (6-8). After 10 days, the microcirculation had returned to normal. At this time, replacement of ERC is reported to be initiated, but CFU still suppressed (6-8). This suggested that the vascular response elicited by Ep was not related to CFU but rather to ERC. That these responses were related to the presence of ERC rather than cells in later stages of differentiation was suggested by the subnormal incorporation of ^{59}Fe at this time. These vascular responses and changes in ^{59}Fe incorporation were abolished in polycythemic animals having no circulating Ep, as also has been reported previously (1-4). Thus, both ERC and Ep are required to elicit these responses, confirming the hypothesis of McCuskey *et al.* (1-5) that the effect of Ep on the microvasculature is indirect and mediated by some substance(s) released from ERC when stimulated by Ep. As a result, the reduction in microcirculation seen 32 days after administration of Myleran suggests that reductions in either ERC or Ep may have been occurring at that time.

Further insight into this mechanism was obtained from the experiments utilizing polycythemic animals treated with Myleran, in which both CFU and circulating Ep were depressed, and where responses to the administration of a split dose of Ep, 3 days apart, could be related to ERC replication (first injection of Ep) or differentiation (second injection of Ep) (7).

The data demonstrate a transient microvascular response within 24 hr after a single injection of Ep. Simultaneous injection of hydroxyurea, which prevents replication of ERC (7), abolished the vascular response. In contrast to the report of Reissmann and Udupa (7), some ERC did differentiate, as indicated by the transient increase in the incorporation of ^{59}Fe 4 days following a single injection of Ep. Administration of a second dose of Ep also was followed, 24 hr later, by a limited microvascular response as well as an immediate differentiation of erythroid cells, as indicated by the increased incorporations of ^{59}Fe in these animals. That

this second response was maintained at a low level, rather than being transient, is suggested by the persistence of a slight elevation in blood flow on Day 3.

These results suggest that the vasoactive property of Ep may be related to its stimulation of ERC to replicate, since the response following a single injection of Ep was seen 24 hr later when only replication of ERC is reported to occur (7). That the unexpected increase in incorporation of ^{59}Fe on Day 4 was not accompanied by any change in microcirculation further supports the conclusion that ERC, rather than erythroid cells in later states of differentiation, are the source of the vasoactive substance(s). The microvascular response seen after the second injection is interpreted as the result of additional ERC being triggered into replication at that time. However, a microvascular response related to the stimulation by Ep of ERC to differentiate cannot be conclusively eliminated because a persistent but limited response was seen after the second injection of Ep. Since the concentration of Ep in the second injection was one-half that of the initial injection and Reissmann and Udupa (7) have demonstrated that the responses of ERC to Ep are dose dependent, a larger response might have been elicited with 8 U of Ep.

The increases in blood flow through the splenic red pulp, induced by a vasoactive substance(s) released from ERC when stimulated by Ep, are suggested to be one of a series of changes in the microenvironment making it conducive for further erythroid differentiation, maturation, and release (1–5, 9, 10).

Summary. Alterations in the splenic microcirculation which accompany the suppression and recovery of CFU and ERC were studied in mice treated with Myleran and in polycythemic mice treated with Myleran and Ep. During the time of suppression of ERC by Myleran in nonpolycythemic mice, blood flow was reduced significantly. Flow returned to normal during the time when repopulation of ERC was occurring but CFU was still suppressed. Administration of a split dose of Ep 3 days apart to polycythemic mice treated with Myleran resulted in dramatic increases in flow 24 hr after the first injection of Ep, with no increases in ^{59}Fe incorporation in the blood. The microvascular response was nearly abolished by 48 hr after this injection. The second injection of Ep elicited limited increases in blood flow 24 hr later, accompanied by increases in ^{59}Fe uptake. These results suggest that the effect of Ep on the splenic microvasculature is indirect and mediated by a vasoactive substance(s) released from replicating ERC following stimulation by Ep.

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