the previously reported inhibition of porcine kidney leucine aminopeptidase by captopril[2]. Leucine aminopeptidase is a membrane-bound protease which has been implicated in the events that induce resting cells to proceed to the proliferative stage[15]. A potent inhibitor of leucine aminopeptidase activity, bestatin, has previously been shown to possess immunomodulatory activity[16,17]. Further experiments, however, are needed to establish whether a relationship exists between the inhibition of this enzymatic activity and the observed effects of captopril on lymphocyte responsiveness.

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Effects of sphingolipids on erythroblastic maturation in the mouse[1]

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Summary. The changes effected by injection of an extract of phospholipids obtained from the plasma of normal human donors (PLE) or an emulsion of commercially available sphingolipids on erythropoiesis in the mouse were studied. The parameters followed were ^{59}Fe uptake by the erythroid tissue and the number of circulating reticulocytes. It was found that in the 12-24-h period following administration of PLE or purified sphingomyelin a significant increase in ^{59}Fe uptake by circulating RBC and by their hemic fraction takes place. This change was associated with a higher ^{59}Fe utilization by the bone marrow and with an increase in the number of circulating reticulocytes.

In the light of the current theory, erythropoiesis in vivo is governed by erythropoietin (Epo)[2,3]. Since this unified theory does not explain many experimental and physiologic situations, the existence of accessory oxygen-independent mechanisms has been postulated[4-10].

Previous reports have indicated that a lipid factor(s) which probably affects erythroblastic cellular maturation, could participate in the quantitative regulation of erythropoiesis[5,6,11-13]. In this study we report the effects of some major phospholipids of human plasma and various commercial sphingolipids on mouse erythropoiesis as measured by ^{59}Fe uptake by RBC, spleen, bone marrow and by the number of circulating reticulocytes.

Material and methods. Preparation of phospholipids. Blood obtained from normal volunteers no less than 6 months from a previous donation, was received in acid-citrate-dextrosa anticoagulant (ACD) and centrifuged at 3000 × g for 30 min. Phospholipid extracts (PLE) were prepared from the plasma using the technique described by Reed et al.[14] for extraction of phospholipids from red blood cells. Methanol and chloroform were used in the same proportions (1:5) as in the original method. A fraction of the dry final product was emulsified in isotonic saline solution, adjusted to a concentration of 2 mg/ml and kept at 4 °C until used. A portion of the dry extract was separated for determination of total lipid phosphorus according to the technique of Fiske and Subbarow[15]. Another fraction was prepared for TLC in silica gel as described by Marinetti et al.[16].

After identification of sphingomyelin and phosphatidyl choline in the chromatograms the spots were marked, cut out and eluted with 0.5 N methanolic HCl as described by Reed et al.[14]. The phosphorus content in aliquots of the eluates was determined and the rest of each eluate was emulsified in isotonic saline solution at a concentration of 2 mg/ml and stored frozen until use.

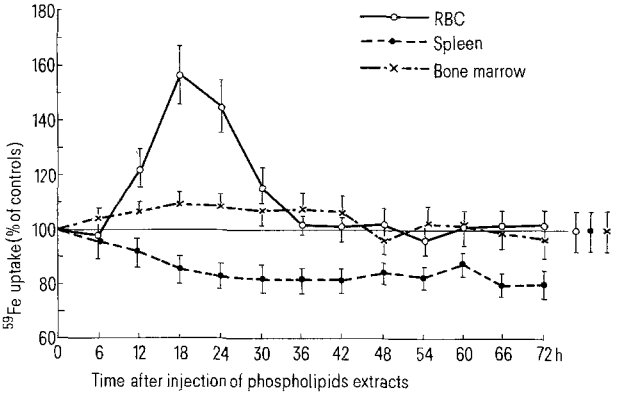

Figure 1. Changes in ^{59}Fe utilization by RBC, —○—; bone marrow —·—·x—·— and spleen ——●——, measured at various times after injection of plasma phospholipid extracts. Values are means ± SE of 8 animals.

Commercial sphingomyelin, phosphatidyl-choline and total bovine brain ceramides were obtained from Sigma Chemical Co., St. Louis (MO, USA). These compounds were emulsified by sonication in isotonic saline solution at a concentration of 2 mg/ml and stored at 4 °C.

We used C3H/FWD F_1 female mice 8–10 weeks of age. Normal unmanipulated mice were injected i.p. with 0.5 ml of the PLE emulsion. Every 6 h from 6 to 72 h after treatment with PLE groups of 8 mice were injected i.v. with 0.25 µCi of ^{59}Fe. 3 h later the percentage of the injected radioiron taken up by the spleen, both femurs and circulating RBC was calculated. RBC were washed twice, the radioactivity counted and the heme extracted as described by Kassenaar et al.[17]. For calculation of RBC ^{59}Fe uptake the blood volume was assumed to be 6% of b.wt. Radioactivity in the heme was expressed in cpm. Controls received 0.85% NaCl in an equivalent volume.

Iron-59 plasma clearance was measured in groups of 10 animals 18 h after injection of PLE. At this time 0.25 µCi of ^{59}Fe was given i.v. 40 min later the animals were bled and the percentage of radioiron remaining in the plasma was calculated.

Groups of mice were made polycythemic by i.p. injection of 1 ml of washed isologous erythrocytes on day O. On day 5, 0.36 cm^3/g of b.wt of carbon monoxide was injected s.c. in order to induce a burst of endogenously formed erythropoietin[18]. At 24 or 48 h later groups of 8 mice were injected i.p. with 0.25 ml of the PLE preparation. 24 h later the animales were given 0.25 µCi of iron-59 and the topographic distribution of the radiodose was measured as described for normal mice. Controls received 0.85% NaCl in a equivalent volume. Reticulocytes were counted.

Results. Figure 1 shows the effect of plasma phospholipid extract on the ^{59}Fe utilization by the spleen, both femurs and circulating RBC in normal mice. A marked rise in ^{59}Fe uptake by RBC, with a peak at 18 h, was observed in the 12–24 h-interval following PLE administration. A smaller though similar change was found in the bone marrow. The splenic area, on the other hand, showed a consistent and protracted reduction in its ability to incorporate radioiron. Significantly lower than normal values ($p < 0.05$) were present up to 72 h after PLE administration.

Table 1 shows the effect of the various phospholipids on ^{59}Fe uptake by RBC and by their hemic fraction measured 18 h after treatment. The chromatograms showed a good separation of major plasma phospholipids when compared with reference sphingomyelin and phosphatidyl choline. When aliquots of the eluates obtained from the spots identified as sphingomyelin were injected in amounts matched by their phosphorus content to the commercial sphingomyelin, they reproduced the effects of both total lipid extracts and commercial sphingomyelin.

The half-times of plasma ^{59}Fe in animals injected with PLE were significantly shorter ($p < 0.01$) than in saline-treated controls (table 2). Reticulocytes counts were increased at 12 and 18 h after PLE injection ($p < 0.01$) (table 3).

The effects of PLE on polycythemic mice injected with CO are shown in figure 2. Iron-59 utilization by circulating RBC, bone marrow and spleen was increased when lipids extracts were administered either at 24 or 48 h after the injection of CO, though the effect was larger at 48 h.

Discussion. Our studies show that in the 12–24 h-period that follows administration of PLE or purified sphingomyelin, a highly significant increase in ^{59}Fe uptake into circulating RBC and into their hemic fraction takes place. This change was associated with an increase in the uptake of ^{59}Fe by the bone marrow and in the number of reticulocytes in the circulation.

The reduction of splenic erythropoiesis might suggest that depletion of the organ as a reticulocyte depot could explain

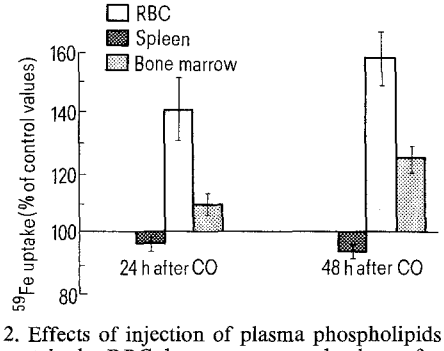

Figure 2. Effects of injection of plasma phospholipids extracts on iron-59 uptake by RBC, bone marrow and spleen of polycythemic mice. Phospholipids were administered at 24 or 48 h after stimulation of endogenous erythropoietin by subcutaneous injection of 0.35 cm^3/g of b.wt of carbon monoxide. Values are means±SE of 8 animals.

Table 1. Iron ^{59}Fe uptake by RBC and into their hemic fraction measured 18 h after injection of various sphingolipids

Material injected	Dose	3 h RBC % iron 59 uptake	Heme ^{59}Fe[d]
Saline (8)	0.5 ml	8.43	1660
Total lipids extracts (9)	3.0 ml[a]	17.24	2870
Sphingomyelin (eluates) (5)	2.0 mg[b]	14.95	2560
Sphingomyelin (9)	2.0 mg[c]	18.09	2460
Phosphatydyl choline (8)	2.0 mg[c]	11.00	-
Brain ceramides (8)	2.0 mg[c]	12.30	-

[a] The dose indicates the volume of plasma from which the injected dose of PLE was obtained. [b] Estimated by the phosphorus content. [c] Commercial purified material. [d] Iron 59 (cpm) in the heme fraction obtained from a 0.5 ml sample of blood. In brackets number of mice. Underlined values differ from control by p of 0.05 or less.

Table 2. Half time of plasma ^{59}Fe clearance in mice, 18 h after administration of PLE or purified sphingomyelin

Groups	Plasma half time ^{59}Fe clearance (min)
Saline (10)[a]	39.4±1.1[b]
PLE (10)	36.2±0.9
Sphingomyelin (10)	35.1±1.0

[a] In brackets number of mice in each group. [b] Mean±SE.

Table 3. Percent of circulating reticulocytes at various times after injection of plasma phospholipids extracts

Material	Retyculocytes[a] (%) Before treatment	6 h	12 h	18 h
Saline	0.9±0.3[b]	0.8±0.1	1.1±0.3	0.8±0.3
PLE	0.8±0.2	0.8±0.3	1.9±0.6	3.1±0.6[c]

[a] Reticulocyte counting in each group was performed before and at 6, 12 and 18 h after treatment with the material used. [b] Mean±SE in groups of 10 mice each. [c] Underlined values differs from control by p of 0.05 or less.

the increase of these elements in the circulation. However, the reticulocyte releasing effect of other stimuli on the spleen occurs whithin a few h[19] i.e., it is early and transient. In contrast the increase in ^{59}Fe uptake and number of circulating reticulocytes appeared with a delay of 12 h and lasted up to 24 h after PLE administration. Replenishing of the reticulocyte pool in the spleen takes only a few h, the observed protracted splenic erythropoietic depression can therefore not be interpreted solely on the basis of a short-lasting reticulocyte depletion.

The association of an increased RBC ^{59}Fe uptake with a shorter half life of plasma radioiron probably reflects a genuine increase in the rate of hemoglobin synthesis and not a mere mobilization of reticulocytes.

This time-course for the changes also makes it improbable that PLE had an Epo-like effect, since this would have demanded a longer time for the effects to be apparent in peripheral blood.

The most plausible interpretation of the effects of phospholipids on the erythropoietic parameters here measured may be based on an action on erythroid precursors younger than the reticulocyte. This interpretation is strongly supported by the effects observed after administration of PLE to polycythemic recipients after CO-Epo stimulation as is shown in figure 2. In this experiments PLE were given at a time when nucleated erythroid precursors in the organs were not beyond the stages of basophilic or earlier polychromatic erythroblasts.

Although the data reported here do not provide direct evidence that lipids act as regulatory factors on erythropoiesis, they suggest an erythropoietic activity of some sphingolipids. A mere nutritional effect of lipids on the growth of red cells appears unlikely since mouse plasma contained presumably non-limiting amounts of sphingolipids prior to injection. These observations also indicate that the erythroblastic compartment is capable of significant changes in the rate of erythrocytic output.

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Enhanced enzyme activity in concentrated salt solutions

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Summary. The enzyme activity of thrombin, β-factor Xa-E and trypsin was enhanced in 1.0 M sodium citrate solution. Thrombin activated factor X. Synthetic substrate S-2222, designed for factor Xa, was hydrolyzed more rapidly with factor Xa or with thrombin in 1.0 M sodium citrate solution than in dilute salt solutions.

In 1949 it was found that prothrombin complex preparations spontaneously generated thrombin in 1.0 M sodium citrate solutions[2]. Other salt solutions such as ammonium sulfate, magnesium sulfate, sodium sulfate, potassium citrate, potassium oxalate and dipotassium hydrogen phosphate had a similar effect[3]. Factor X converted to factor Xa by autocatalysis. It may be that many enzyme reactions progress more rapidly in certain strong salt solutions, and in support of that idea we are reporting on a study of thrombin, β-factor Xa-E, and trypsin. All three had their activity enhanced in 1.0 M sodium citrate solution. For the experiments all of our proteins were single components as measured by polyacrylamide gel electrophoresis, and were prepared and assayed by methods developed and described in this laboratory.

In physiological saline solution slow conversion of factor X to factor Xa occurred with thrombin. Only 8% of the potential activity generated in 2 h (fig. 1). This low yield is in accord with the general view that factor X is not activated by thrombin. Complete activation was, however, achieved in 1.0 M sodium citrate solution. Using the same quantities of factor X and thrombin in 3 M ammonium sulfate solutions also fulfilled conditions for accelerated activation but not as well as was the case with sodium citrate. The bond broken by thrombin is in the sequence Arg: Ile-Val-Gly-Gly-Arg-Asp. Commonly the bond bro-