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Release of Erythropoietin From Macrophages by Treatment With Silica

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An erythropoietic stimulating factor (ESF) can be shown to be released from preincubated macrophage-containing cell suspensions from mice by the macrophage-specific, cytotoxic agent, silica. A concentrated silica-treated spleen cell supernatant containing ESF is shown to cause a dose-dependent increase in ^{59}Fe incorporation into red blood cells using the in vivo polycythemic mouse bioassay. The ESF from the same supernatant can also be neutralized by anti-erythropoietin. A second concentrated supernatant fractionated using wheat germ lectin-Sepharose 6MB and compared to either unfractionated or fractionated step III erythropoietin (Ep), tested in vitro using the erythroid colony-forming technique and 12-day fetal liver as target cells, indicates parallelism of all linear dose-response lines. This, together with the in vivo data, strongly suggests that the ESF released from macrophages treated with silica is, in fact, Ep. Substituting Ca^{2+} ions for fetal calf serum in the preincubation procedure results in the same activity being released compared to the presence of 1% or 20% fetal calf serum.

Key words: erythropoietin, macrophages, silica, erythrocytic colony-forming units, polycythemic mouse bioassay, anti-erythropoietin

Crystalline silica (silicon dioxide, SiO_2) has been shown to be specifically cytotoxic for macrophages [1, 6]. We [12, 13] have recently demonstrated that an erythropoietic stimulating factor (ESF) can be released when cell suspensions from 14-day fetal liver and adult bone marrow and spleen are preincubated with silica. In addition, it was shown that the activity of the ESF released was dependent on the erythropoietic status of the animal [13]. The ESF was detected using the erythroid colony-forming technique [17] and 12-day fetal liver as a source of erythrocytic colony-forming units (CFU-E). It has been previously shown that 12-day fetal liver CFU-E are extremely sensitive to erythropoietin (Ep), thus providing an in vitro assay for this hormone [10, 11].

In this communication we extend our previous results by attempting to show that the ESF released from macrophages after treatment with silica is Ep, as demonstrated by its effect in vitro and in vivo, and by its neutralization by anti-Ep. In addition, evidence is presented showing that release of Ep can be obtained under essentially serum-free conditions.

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METHODS

Female CBA/Ca and C57/65J mice were used. Preparation of single cell spleen suspensions has been described elsewhere [10]. However, for clarity, spleens were first homogenized in a loosely fitting glass homogenizer using Hank's balanced salt solution (BSS) containing 3% fetal calf serum (FCS). After the large debris sank to the bottom the suspension was decanted into a plastic tube (Falcon Plastics, Oxnard, CA) and again left on ice for about 5 min. Thereafter, the suspension was withdrawn using a 25-gauge needle and the volume measured. Nucleated cells were counted (Coulter Counter Model ZF).

Treatment of cells with silica has been detailed previously [13]. In summary, 8×10^6 spleen cells/ml were preincubated with 1×10^{-4} gm/ml silica (Min-U-Sil; particle size 2–5 μm; Whitacker, Clark & Daniels, Plainfield, NJ) for 30 min at 37°C in 5% CO_2. Cells were normally suspended in Hank's BSS containing 3% FCS. Thereafter, the cells and silica were centrifuged down at 1,500 rpm at 4°C, and the supernatant was used as a source of erythropoietic stimulating material.

Two mass cultures of silica-treated spleen supernatant were prepared. Batch 1 employed 20 C57Bl mouse spleens, which produced 450 ml, and batch 2 used 25 C57Bl mouse spleens and gave rise to 550 ml of supernatant. Control spleen suspensions were set up without silica addition. The supernatants from both batches were concentrated down to 50 ml using Sephadex G-25 medium grade (Pharmacia). The concentrate was dialysed against 4 changes of double-distilled water in 24 h and lyophilized.

Batch 1 was dissolved in 25 ml physiological saline and assayed in the polycythemic mouse bioassay. For this, virgin Swiss Webster mice (about 22 gm) with 5 mice/group were used after exposure to hypoxia at 0.4 atm for 19 h/day for 3 weeks. Animals having a hemocrit below 65% were not used. The mice were injected intraperitoneally with the samples on days 5 and 6 post-hypoxia, followed by intravenous injection of radioiron on day 7. The ^{59}Fe uptake into red blood cells was determined 72 h later. Batch 1 was also used to test if the activity was Ep by reaction with antierythropoietin (anti-Ep). Antiserum was prepared in rabbits by injection of crude human urinary Ep. One milliliter of the sample was assayed alone or after treatment with anti-Ep (capable of neutralizing 0.6 IU Ep) for 1 h. To prevent carry-over of anti-Ep into assay mice, the reaction was treated with goat anti-rabbit gamma globulin (GARGG) and the precipitate removed before injection. As control, GARGG was also added to the untreated sample, along with normal rabbit IgG.

Batch 2 of the lyophilized silica-treated spleen supernatant was dissolved in 2 ml of degassed phosphate buffer (see below). Of this, 1.5 ml were passed over a wheat germ lectin-Sepharose 6MB (Pharmacia) affinity chromatography column, which had first been equilibrated with 10 column volumes (1 column volume was equivalent to 2.6 ml) of PBS. All material that remained unbound to wheat germ lectin was eluted with 5 column volumes of PBS alone. Bound material was eluted with 8 column volumes of PBS containing 0.1 M N-acetyl glucosamine. All fractions of unbound or bound material were pooled, dialysed for 24 h against 4 changes of double-distilled water, and lyophilized. The lyophilized unbound and bound pooled fractions were then reconstituted with physiological saline in the same volume as was applied to the column; namely, 1.5 ml. The same fractionation procedure was used for step III Ep (Connaught Laboratories, Ontario, Canada), but in this case 2 ml containing 100 units was added to the column.

Protein determinations for all fractions were performed using a modification [18] of the Lowry procedure employing a standardized serum (Kontrollogen-L, Behringwerke, West Germany).

The phosphate-buffered saline (PBS) used for both chromatography and other experiments described in the Results was prepared according to Rabinowitz [8], but without Ca^{2+}, Mg^{2+}, EDTA, and glucose. The pH was 7.2.

Twelve-day fetal liver cell suspensions [10] were prepared by passing the dissected out organs through decreasing diameter syringe needles from 18 gauge to 22 gauge, and finally 25 gauge, in Hank's BSS containing 3% FCS. Six organs were suspended in 1 ml medium. Cells were counted as described above.

The erythroid colony-forming technique using 12-day fetal liver CFU-E as target cells for detecting erythropoietic stimulating activity in the supernatants was similar to that described previously [11]. Experiments were performed at least 2 times in duplicate wells containing 0.5 ml of the culture components. Colonies were enumerated using an inverted microscope. At optimal (75 mU Ep/ml) Ep concentrations, the number of fetal liver CFU-E colonies obtained is approximately 3,500 CFU-E/10^5 cells.

Regression analysis of the normalized in vitro dose-response curves has been described previously [11]. Probit analysis of the dose responses was performed using the BMD 03S program from the Health Sciences Computing Facility, UCLA. In addition, a chi-squared test was used to examine the degree of association of the points on the probit line.

RESULTS

Table I shows the effect of injecting 0.4 ml, 0.8 ml, and 1.6 ml of the C57Bl concentrated silica-treated spleen supernatant (batch 1) into polycythemic mice. Increasing volume of this supernatant results in a corresponding increase in the ^{59}Fe incorporation, thus providing evidence that the dose response so obtained is similar to that obtained when Ep is injected.

Controls – ie, suspensions incubated without silica (not shown in the diagram) – consistently gave ^{59}Fe incorporation readings below the 50 mU/ml level, the lowest concentration that can be detected using this in vivo polycythemic mouse bioassay.

Table II demonstrates that anti-Ep can almost completely neutralize the erythropoietic stimulating effect shown in Table I. GARGG is used to neutralize the effect of anti-Ep action after it has reacted with the sample (and Ep as control), and therefore to eliminate the effect of antiserum in the assay mouse.

TABLE I. Effect of the Concentrated Silica-Treated Spleen Supernatant (Batch 1) When Injected Into Polycythemic Mice*

Material assayed	% RBC ^{59}Fe incorporation (mean ± SEM)
Saline	0.3 ± 0.05
0.2 IU Ep	3.92 ± 0.42
0.4 IU Ep	8.14 ± 0.65
0.4 ml sample	2.06 ± 0.29
0.8 ml sample	4.14 ± 0.62
1.6 ml sample	10.43 ± 1.12

*Similar volumes of the supernatant obtained from cells incubated without silica produced values in ^{59}Fe incorporation not significantly different from the saline control.

An additional attempt to show that the ESF present in the silica-treated spleen supernatant was similar to Ep, was to purify the factor and compare the in vitro dose-response curve with similarly treated step III Ep. Figure 1 shows the dose-response curves for unfractionated step III Ep and the original, concentrated batch 2 supernatant. Also shown are the dose-response curves for the wheat germ lectin unbound and bound fractions for both step III and batch 2 supernatant. As shown in Figure 1, the majority of the protein material remains unbound to wheat germ lectin and is eluted using PBS alone. In the case of EP, some of the activity was eluted in this first peak and is probably due to overloading of the column. The second peak of eluted bound material contains most if

TABLE II. Effect of Reacting the Concentrated Silica-Treated Spleen Supernatant With Anti-Ep. Tested in the Polycythemic Mouse Bioassay

Material assayed	% RBC ^{59}Fe incorporation (mean ± SEM)
Saline	0.53 ± 0.08
0.4 IU Ep + GARGG + normal rabbit serum	9.32 ± 1.47
0.4 IU Ep + Anti-Ep IgG + GARGG	0.58 ± 0.10
1 ml sample + GARGG + normal rabbit serum	7.22 ± 1.30
1 ml sample + anti-Ep IgG + GARGG	0.23 ± 0.05

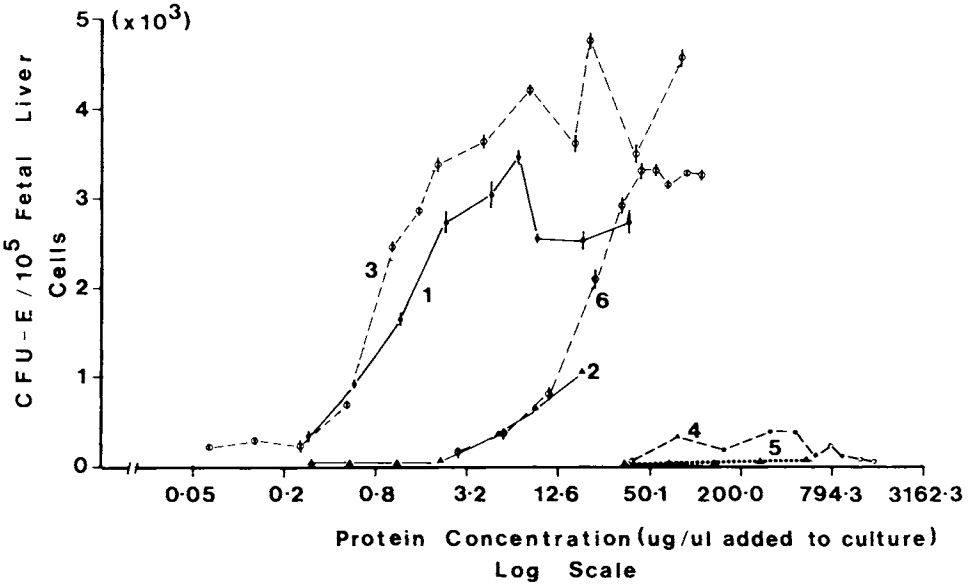

Fig. 1. Comparison of dose-response curves for step III Ep and the concentrated silica-treated spleen supernatant (batch 2) before and after fractionation on wheat germ lectin-Sepharose 6MB. Curve 1, original, unfractionated step III Ep (4.6 μg protein/μl); curve 2, fractionated step III Ep, unbound peak. (2.15 μg proteins/μl); curve 3, fractionated step III Ep, bound peak. (1.02 μg protein/μl); curve 4, original, concentrated supernatant (batch 2) (6.1 μg protein/μl); curve 5, fractionated, unbound peak (batch 2) (2.65 μg protein/μl); curve 6, fractionated, bound peak (batch 2) (0.44 μg protein/μl); two experiments performed.

not all the activity. With regard to the Ep fractionation, the dose response shows a modest additional stimulation of the fetal liver CFU-E, and a plateau at optimal concentrations is obtained. When batch 2 supernatant was passed through the column, almost all the activity was found in the wheat germ lectin bound material, and it also shows a plateau.

That the erythroid colony-forming technique can be used as a specific in vitro assay for Ep is shown in Figure 2. Here, the results for dose-response curves 1, 3, and 6 of Figure 1 have been transformed into percent CFU-E calculated from the maximum number of CFU-E obtained and plotted against the log protein concentration. In this form, a dose-response line for an unknown Ep preparation (in this case the silica-treated, concentrated, and fractionated spleen supernatant) must produce a dose response parallel to that obtained for a known Ep preparation – ie, step III Ep. Examination of the regression coefficients (Fig. 2) indicates similarity between those for both Ep preparations and the fractionated supernatant. Regression analysis substantiates this within 95% confidence limits. The horizontal displacement to the right for the silica-treated spleen supernatant dose response indicates an Ep preparation of lower potency than that for step III Ep. A concentration 12.8 times higher than that for step III Ep is required in order to stimulate 50% of the potential CFU-E present – ie, the essential dose 50 (ED_{50}) obtained by probit analysis.

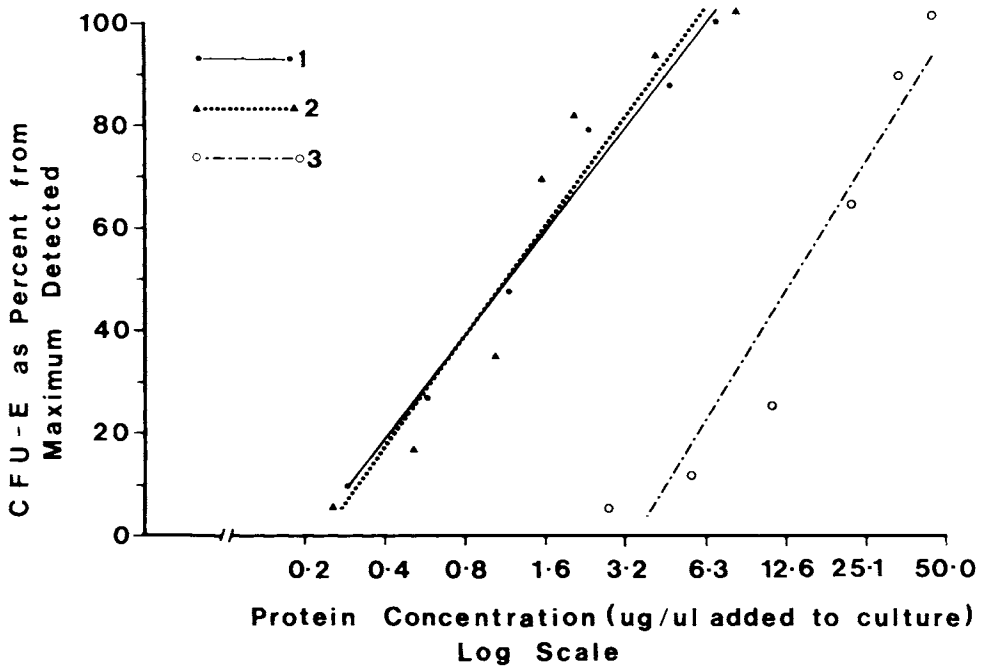

Fig. 2. Comparison of linear dose-response lines for 1) original, unfractionated step III Ep. Linear regression: $y = 45.6 + 67.4(x)$; $r = 0.99$; $P < 0.001$. Probit line: $4.9 + 2.3$ (x); $P < 0.001$; 2) Fractionated step III Ep, bound peak. Linear regression: $y = 45.9 + 72.1$ (x); $r = 0.97$; $P < 0.05$. Probit line: $4.9 + 2.3$ (x); $P < 0.05$; 3) fractionated, bound peak (batch 2). Linear regression: $y = -45.2 + 74.5$ (x); $r = 0.96$; $P < 0.05$. Probit line: $1.5 + 3.1$ (x); $P < 0.05$; two experiments performed.

Preincubation of macrophage-containing cell suspensions with silica is routinely performed using 3% fetal calf serum added to the suspension culture. Since this is a very low concentration of fetal calf serum, it was of interest to know whether the reaction would occur in serum-free conditions or whether certain cations were necessary. To investigate this, CBA/Ca spleen cell suspensions were incubated either 1) without fetal calf serum or with different concentrations of fetal calf serum or 2) with 2 mM Ca^{2+} ($CaCl_2$) and with 2 mM Ca^{2+} plus different concentrations of fetal calf serum added to PBS.

Table III shows the effect of incubating spleen cell suspensions in PBS alone or with different concentrations of fetal calf serum and/or 2 mM Ca^{2+}. When 1% fetal calf serum is added to PBS, an enhancement in CFU-E growth is observed compared to that incubated in the absence of fetal calf serum (330 ± 20 compared to 220 ± 20). Increasing the concentration of fetal calf serum from 1% to 20% does not affect the inital enhancement. When 2 mM Ca^{2+} is added to PBS in the absence of fetal calf serum, a similar increase in the number of CFU-E is obtained over that found in the presence of silica in PBS alone (350 ± 20 compared to 220 ± 20). The increase in CFU-E observed with 2 mM Ca^{2+} alone (350 ± 20) is the same as that obtained when fetal calf serum concentrations from 1% to 20% are added to the PBS. The addition of fetal calf serum to PBS containing 2 mM Ca^{2+} slightly increases the number of CFU-E over the number obtained for fetal calf serum on 2 mM Ca^{2+} alone. Increasing the serum concentration from 1% to 20% does not have an effect on the activity released into the supernatant after the spleen cells have been treated with silica.

DISCUSSION

A previous report demonstrated that an erythropoietic stimulating factor (ESF) could be shown to be released into the extracellular fluid when fetal liver and adult bone marrow and spleen cell suspensions were preincubated with silica [13]. In this communication we have cited evidence that the ESF is almost definitely erythropoietin (Ep), with the in vivo polycythemic mouse bioassay, its neutralization by anti-Ep and its similar effect to Ep in vitro as substantiation for our hypothesis. In addition, the ESF activity released by silica from macrophages is dependent on the erythropoietic status of the animal, indicating that the ESF activity can be manipulated in a similar manner to that of plasma Ep [13]. Moreover, release of ESF is actinomycin D sensitive both in normal and anemic mice [14], a result similar to that obtained by Schooley and Mahlman [15] in their investigations on extrarenal Ep production in lead-poisoned rats. Thus all data point to the macrophage-released ESF being Ep.

The in vitro dose-response effects of macrophage-released ESF deserve an additional comment. Recent work by Spivak [16] showed that Ep could be separated from other protein components using affinity chromatography on wheat germ lectin covalently bound to Sepharose 6MB. Both wheat germ lectin and Ep have N-acetyl glucosamine residues, so that the lectin binds Ep and can be eluted with this sugar. The results presented in Figure 1 agree very well with those of Spivak et al [16] and therefore provide an easy method of removing many of the toxic substances from commercially obtained Ep and human urinary Ep. The fact that the ESF also binds to wheat germ lectin indicates that the molecule also contains the required sugar moiety. It does not, however, indicate that the substance is Ep. We [11] and others [4] have shown that when raw data from CFU-E dose-response curves are normalized and transformed as indicated in Figure 2, different Ep

TABLE III. The Effect of Fetal Calf Serum or 2 mM Ca^{2+} Alone or in Combination on the Release of Ep From Silica-Treated Spleen Cells

	12-Day fetal liver CFU-E/10^5 cells[a]			
	Fetal calf serum alone		Fetal calf serum + 2 mM Ca^{2+}	
Serum concentration (%)	Control ± SEM	After silica ± SEM	Control ± SEM	After silica ± SEM
0	110 ± 10	220 ± 20	155 ± 15	350 ± 20
1	140 ± 10	330 ± 20	160 ± 20	500 ± 10
2.5	155 ± 5	365 ± 15	145 ± 15	455 ± 27
5	160 ± 10	375 ± 5	160 ± 20	455 ± 5
10	185 ± 15	355 ± 15	150 ± 20	425 ± 25
20	145 ± 15	370 ± 10	165 ± 15	445 ± 25

[a]Results are expressed as the mean ± SEM of 4 replicates.

preparations acting on the same target cell must result in parallel linear dose-response lines. These dose-response lines can be horizontally displaced, showing that the Ep preparations tested have different relative potencies or specific activities. This is the case when normal or wheat germ lectin-purified step III Ep is compared to the concentrated, wheat germ lectin-purified silica-treated spleen supernatant. This again indicates that the silica-treated spleen supernatant contains Ep.

Release of Ep from macrophages by treatment with silica takes place in the presence of serum. That fetal calf serum is necessary in the preincubation procedure is shown in Table III. Also shown is that a fetal calf serum concentration as low as 1% is required, increasing concentrations having no additional effect. In addition, fetal calf serum can be replaced by 2 mM Ca^{2+}. Addition of 1% fetal calf serum caused a slight increase in released activity. However, increasing this concentration to 20% again did not result in any significant increase (or decrease) in released activity. The possibility therefore arises of obtaining a native Ep preparation that does not contain the protein contamination usually present in both commercial and human urinary Ep.

The fact that macrophages can release Ep indicates an important role for this cell in erythropoietic differentiation. This comes from the presence in both the fetal liver and adult bone marrow of the so-called blood islands. The blood islands consist of a central macrophage-like cell with differentiating erythroblasts surrounding it. We [10] have postulated that the release of only minute amounts of Ep from macrophages could be high amounts for the differentiating cells in the immediate vicinity or even in association with these centrally lying cells. It is known that macrophages can release a wide range of different factors into the medium. Thus, when macrophages (and perhaps other cell types) are present in hemopoietic cultures either with or without the addition of known stimulating factors (eg, CSF, Ep), it is necessary to know whether these cells are releasing similar factors or other factors, which could result in the production of cells from one or several different pathways of differentiation.

An important implication raised by macrophage-released Ep is that it would provide an explanation for the spleen being a favorable environment for differentiating erythroblasts when the animal is subjected to erythropoietic stimulus, particularly hypoxia. Earlier

work by Rambach et al [9] and Zangheri et al [19] showed that spleen extracts could cause an increase in erythropoiesis. This work led to the postulation that the spleen could be an important extrarenal site for Ep production. Gruber et al [3] showed that fetal liver macrophages are responsible for Ep production during hepatic erythropoiesis since the normal Ep production site, the kidney, has not developed this function at this stage of development. Almost all investigations concerned with the apparent production of Ep from extrarenal sites have laid emphasis on the adult liver. Considering the fact that early hepatic erythropoiesis plays such an important role in the development of the erythropoietic system, it is not surprising that a remnant of this role be carried over into adult life. The same argument can be applied to the spleen, since erythropoiesis is observed in the spleen before being fully transferred to the bone marrow [2, 5, 11].

Besides the study of Gruber et al [3], Peschle et al [7] showed that the extrarenal Ep source in the adult liver was a function of the reticuloendothelial system. Since crystalline silica is specifically cytotoxic for macrophages [1, 6], release of Ep can be shown to be a function of this cell not only in the fetal liver, bone marrow, and spleen [13] but also in the lung, peritoneum, and adult liver [14]. The finding that all macrophage-containing cell suspensions tested can release Ep activity would tend to argue for a ubiquitous production and/or storage phenomenon. Recent experiments in our laboratory utilizing gas-permeable Teflon foils with hydrophobic surfaces on which spleen cells are grown, indicate that Ep can in fact be produced by these cells for at least 4 weeks in culture [Rich, Heit, and Kubanek, in preparation].

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REFERENCES

1. Allison AC, Harrington SS, Birbeck M: J Exp Med 124:141, 1966.
2. Cole RJ, Regen T, White SL, Cheek EM: J Embryol Exp Morphol 34:575, 1975.
3. Gruber DF, Zucali JR, Mirand EA: Exp Hematol 5:392, 1977.
4. Gregory CJ, Tepperman AD, McCulloch EA, Till JE: J Cell Physiol 84:1, 1974.
5. Metcalf D, Moore MAS: "Hemopoietic Cells." Amsterdam: North Holland Publishing Company, 1970.
6. Pearsall NN, Weiser R: J Reticuloendothel Soc 5:107, 1968.
7. Peschle C, Marone G, Genovese A, Rappaport IA, Condorelli M: Blood 47:325, 1976.
8. Rabinowitz Y: Blood 23:811, 1964.
9. Rambach WA, Alt HL, Cooper JAD: Proc Soc Exp Biol Med 108:793, 1961.
10. Rich IN, Kubanek B: J Embryol Exp Morphol 50:57, 1979.
11. Rich IN, Kubanek B: J Embryol Exp Morphol 58:143, 1980.
12. Rich IN, Anselstetter V, Heit W, Kubanek B: J Supramol Struct Suppl 4, 1980.
13. Rich IN, Heit W, Kubanek B: Blut 41:29, 1980.
14. Rich IN, Anselstetter V, Heit W, Kubanek B: In Lucarelli B, Gale RP, Fliedner TM (eds): "Proceeding of the First International Symposium on Fetal Liver Transplantation." Amsterdam: North Holland Publishing Company, 1979, p 95.
15. Schooley JC, Mahlman LJ: Blood 43:425, 1974.
16. Spivak JL, Small D, Shaper JH, Hollenberg MD: Blood 52:1178, 1978.
17. Stephenson JR, Axelrad AA, McLeod DL, Shreeve MM: Proc Natl Acad Sci USA 68:1542, 1971.
18. Wang CS, Smith RL: Anal Biochem 63:414, 1975.
19. Zangheri EO, Fora-De-Moraes F, Lopez OI, Marios I: Experientia 29:706, 1973.