Kor J Physiol (1992) 26 (1), 37-44

Characteristics of Synthesized Red Cells Using Bovine Hemoglobin as Oxygen Carrier

Eng Haeng Cho, Jong Sik Hah and Ku Ja Kim

Department of Physiology, College of Medicine, Ewha Womans University, Seoul, Korea.

= ABSTRACT =

The encapsulation of the purified bovine hemoglobin with phospholipids obtained from egg yolk was performed using a rotary vacuum evaporator. The prepared Hb-containing liposome (hemosome) had good properties as artificial red blood cell. The shape and size of the hemosomes were measured by a phase contrast microscope and image analyzer. The function of the hemosome as oxygen carrier was tested by measuring oxygen saturation curve with blood gas analyzer and infusing it into rats. The prepared hemosome was 1.184 + 0.423 μm in diameter and round in shape. P_{50} value of the hemosome solution was 28 mmHg. The synthesized red cells seem to function as oxygen carrier, because the severely bled rats were prolonged in their life by transfusion of the hemosome solution containing bovine hemoglobin.

Key Words: Hemosome, Artificial red cell, Blood substitute, Encapsulation of hemoglobin.

INTRODUCTION

In the past century or so, blood replacement therpy has been an important problem to life under conditions brought about by severe blood losses. The most widely used applications has been transfusion of the whole blood and blood compoments, but blood transfusion often encountered with problems such as hemolytic reactions, allergic reactions, transmission of diseases and so on. In many cases, appropriate supply of needed blood type is not available.

However the red-cell-free hemoglobin (Hb) solution deliver oxygen to tissue (Amberson et al, 1934), they cause abnormal oxygen saturation, transient kidney damage and vasoconstriction, and exhibit too short life time in circulation (Djordjevich & Miller, 1980; Lieberthal et al, 1987).

Perfluorocarban solutions also has been investigated vigorously as a synthetic blood replacement. But they required the hyperoxia to improve oxygen delivery to tissue, and decreased hepatic energy charge level, distal diastolic coronary pressure and serum proteins including albumin, globulin and fibrinogen (Christensen et al, 1988; Lutz & Stark, 1987; Ozaki et al, 1989; Tuliani et al, 1988).

The encapsulation of hemoglobin in liposome is an interesting means of obtaining blood-substitute because of the following reasons: (1) Hemoglobin and phospholipids are biomolecules which are non-toxic and non-immunogenic materials (Gaber et al, 1983; Djordjevich & Miller, 1980). (2) They provide a useful RBC model for studying the interaction between hemoglobin and lipid layers (Szebeni et al, 1984). (3) Microencapsulation should result in a longer circulation life than red-cell- free hemoglobin. (4) They can be provided easily from bovine RBC and egg yolk.

In the present study, we performed the encapsulation of the bovine-Hb with egg-yolk-phospholipid, and examined some of its pro-

perties as oxygen-carrier in the living animal bodies.

METHODS

Phospholipid was prepared from fresh egg yolk and hemoglobin from bovine and human RBC as previously described (Hah et al, 1990). Hemolysate from fresh bovine blood and outdated human blood which was stored under blood-banking conditions were used for purification of hemoglobin. Bovine blood was purchased from a local slaughter-house just before use. Hemoglobin solution was concentrated to 30% by dehydration through dialysis membrane with sugar.

Phospholipid components were assayed by phosphate measurement in the spots separated using standard TLC methods. The phospholipid used for the hemosome synthesis contained the same amount of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as major constituents and a very small amount of other lipids. α-Tocopherol (10%) was added to the phospholipids to prevent oxidation. The concentrated hemoglobin and phospholipid have been kept in deep freezer (-70°C). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyze the crystallized bovine and human hemoglobin.

Hemosomes were prepared according to the modified film methods of Djordjevich & Miller (1980) and Szebeni et al (1985). After encapsulation, the hemosomes were suspended in Tris-buffered saline (pH 7.4) and sonicated in a bath sonicator (Mettler Electrics, USA) for 20-30 minutes. The free hemoglobin was removed by dialysis against saline (pH 7.4) for overnight using a home-made filter paper bag. Final volume was made to about 10ml per each g of hemoglobin used for encapsulation. This hemosome solutions were rediluted with Tris-buffered saline (pH 7.4) about to 100 times and used for determination of hemoglobin oxidation during incubation at 37°C for 24 hours under mild agitation. Then, the absorbances at the wavelength of 560nm, 577nm and 630nm were recorded on a fluorospectrophotometer (Hitachi 557, Japan), and the percentages of oxyhemoglobin, methemoglobin and hemichrome were calculated from the equations of Szebeni et al (1984) and the absorbances at the wavelengths. The base-line absorbance at 700nm was substracted from the absorbances at the above wavelengthes.

For morphological analysis, hemosome was photographed under a phase-contrast microscope (Laborlux S. Leitz Co., West Germany) and measurements of size distribution in cross-section area, diameter, perimeter and roundness of hemosomes in the photograph were carried out with a image analyzer (Quantimet 520, Cambridge instrument Co., UK). Oxygen-saturation curve was determined by using blood-gas analyzer (Stat profile 3, NOVA Co., USA) as described previously (Hah et al, 1990). The Hill-coefficient (n) was calculated from equation: $\log (S/[100\text{-}S]) = n(\log P_{O_2} - \log P_{50})$, wherein S is the percentage of oxigen saturation (van der Plas et al, 1988). Hemosome solution was saturated with oxygen by bubbling with mixed gas as in the previous study. The mixed gas contained 40mmHg of carbon dioxide and the temperature was maintained in the blood-gas analyzer at 37°C + 0.1°C. pH was stabilized at 7.4 by controlling P_{CO_2} and temperature. In transfusion study, after the rats (300-350g) were anesthetized with sodium pentobarbital, about one-third of the total blood was removed and the same volume of prepared hemosome solution was infused slowly through jugular vein. The total blood of rat was determined as 6% of body weight according to Courtice (1943). Kanamycin was injected by 40mg per 100g body weight after operation for preventing infection and the number of survivors from among the hemosome-transfused rats was observed.

RESULTS

Hemoglobin crystallization

The prepared Hb solutions were examined

on SDS-PAGE. The migration distances of hemoglobins purified from human and bovine RBC were of the same through 15% slab-gel and their molecular weight was 14500 dalton (Fig. 1). When the sample was overloaded with 200 μg per a lane, other proteins also appeared. These other minor protein components appeared to be slightly different between human and bovine hemoglobins on 10% SDS-PAGE (data not shown). Crystallized hemoglobin solutions from each source showed no coagulating activity.

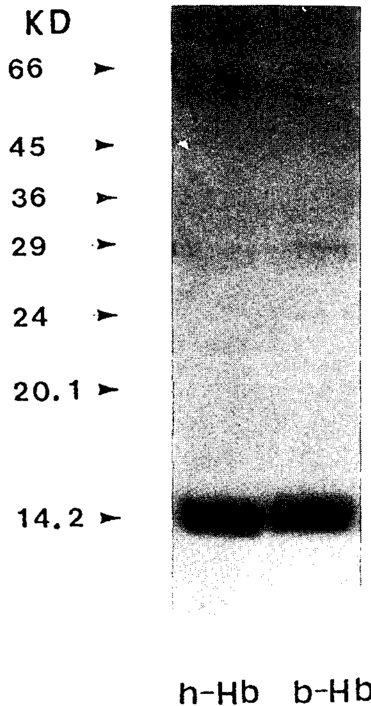

Fig. 1. SDS-PAGE of human and bovine hemoglobin. These hemoglobins were crystallized as described in the text and 10 μg of each protein was loaded per a lane.

Size and shape

The hemosomes appeared to be spherical in structure and mostly separated from one another except partially aggregated (Fig.2). Unlike normal RBC (8 μm in diameter), the prepared hemosomes had a range in diameter from 0.2 to 10 μm. Table 1 shows the results of image analyzer measurements on the hemosome before and after sonication. The average diameters were 1.717 + 0.584 μm in the hemosome before sonication and 1.184 +

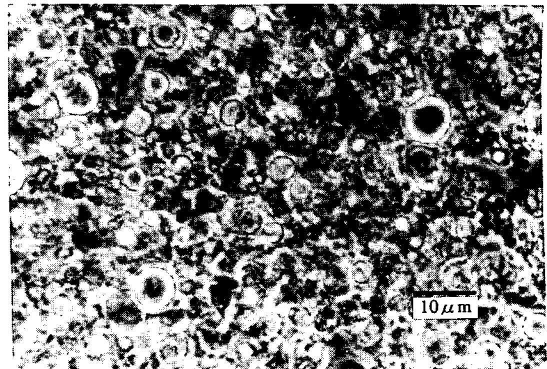

Fig. 2. The phase-contrast microscopic photograph of hemosomes containing bovine hemoglobin before sonication.

Table 1. Morphological characteristics of hemosomes measured by image analyzer before and after sonication

Hemosome Characteristics	Before sonication	After sonication
cross-section area. (μm^2)	1.731 ± 1.489	0.919 ± 0.714
diameter (μm)	1.717 ± 0.584	1.184 ± 0.423
Perimeter (μm)	5.019 ± 1.889	3.336 ± 1.339
Roundness shape	1.332 ± 0.195	1.344 ± 0.154

Results are expressed as the mean + SD

A

LIMITS		COUNT
0.081 -	0.162	0
0.162 -	0.324	13
0.324 -	0.649	123
0.649 -	1.297	198
1.297 -	2.594	105
2.594 -	5.189	60
5.189 -	10.378	11
10.378 -	20.755	2
20.755 -	41.511	0
41.511 -	83.022	0
83.022 -	166.043	0

B

LIMITS		COUNT
0.081 -	0.162	0
0.162 -	0.324	70
0.324 -	0.649	180
0.649 -	1.297	100
1.297 -	2.594	42
2.594 -	5.189	11
5.189 -	10.378	2
10.378 -	20.755	0
20.755 -	41.511	0
41.511 -	83.022	0
83.022 -	166.043	0

Fig. 3. The distribution of hemosomes before(A) and after(B) sonication in count vs. cross-section area. The limits mean ranges of area of the hemosome profile in Fig. 2 and are calibrated in μm^2.

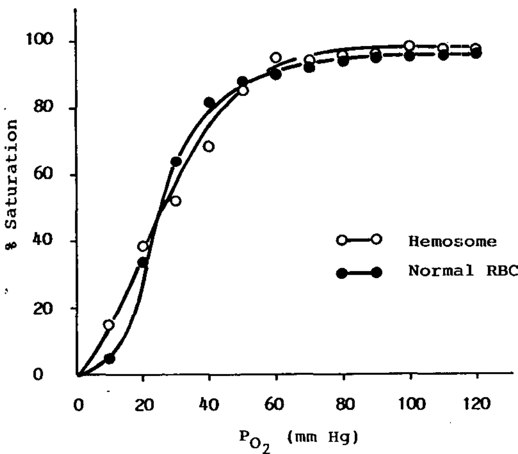

Fig. 4. Hemoglobin-oxygen saturation curve for the bovine HB-containing hemosome and the normal RBC.

0.423 μm after the treatment. The ranges of the area in the hemosome profile in Fig.2 are exhibited at Fig. 3. Sonication reduced the size of hemosomes prepared by a rotary vacuum evaporator. The hemosomes appeared to be elastic structure because it was observed that hemosomes 10 μm in diameter passed through 5 μm Millipore filter by squeeze.

Hemoglobin-oxygen saturation curve

The ability of hemosomes to transport oxygen was determined by their oxygen-saturation measurements. The results of the hemosome containing bovine Hb were compared with those of the normal blood in Fig.4. The hemosome curve was somewhat less sigmoidal than the normal RBC curve. However, the oxygen pressure on 50% oxygen saturation (P_{50}) for the hemosome solution was 28mmHg which

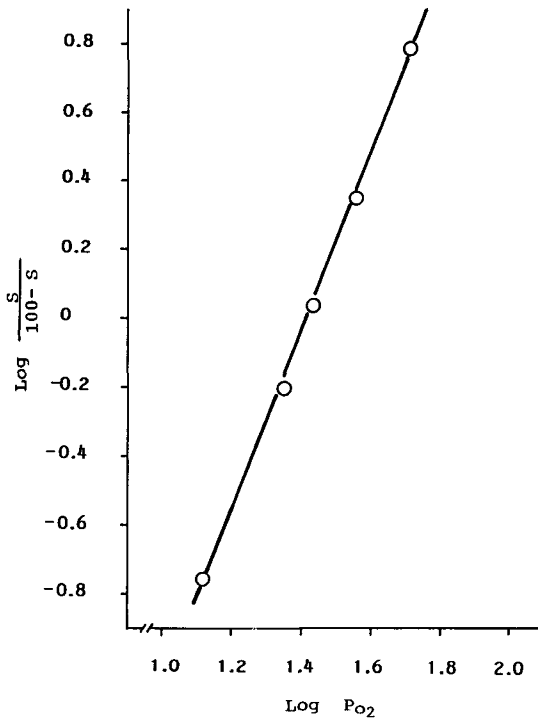

Fig. 5. Hill plot of the bovine Hb-containing hemosomes. Hill-coefficient(n) is 2.63, and S means percentage of oxygen saturation.

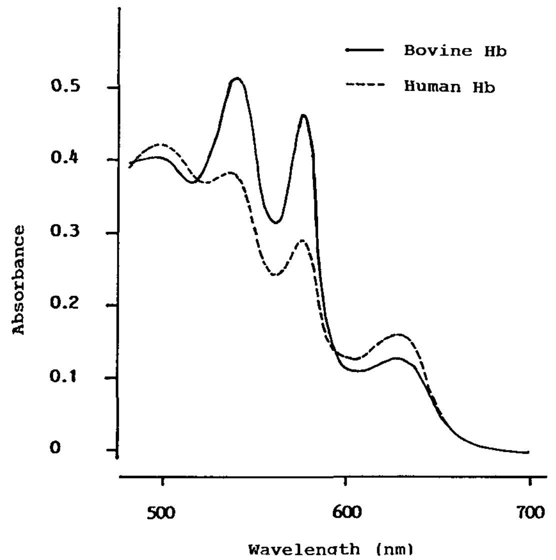

Fig. 6. The visible spectra of the two crystallized hemoglobin(cHb). All absorbances were corrected by the subtraction of the baseline absorbance at 700nm.

was similar to that of the normal RBC. From the hemoglobin-oxygen saturation curve, Hill plot was performed. Hill-coefficient (n) was 2.63 as shown in Fig. 5. This was well matched to the value calculated from the equation of van der Plas et al (1988).

Hemoglobin oxidation in hemosomes

The typical spectra of the two crystalized human and bovine hemoglobins recorded over the range of 500-700nm are shown in Fig.6 and solution of fresh hemosome also showed identical spectral pattern with that of hemoglobin solutions, with absorption maxima at 540nm, 577nm and 630nm and absorption minima at 510nm, 560nm and 600nm. During the hemosome solutions were incubated at 37°C for 24 hours, all the distinguishable spectral peaks disappeared gradually. This was thought to be a result that hemoglobin in the hemosomes was oxidized irreversibly during incubation. The percentage of Hb-oxidation derivatives in the hemosomes determined from the spectral changes is given in Table 2.

Hemosome transfusion

Infusion of bovine Hb-containing hemosome solution into the bled rats were performed. After hemosome infusion, the color of the skin in the mouth, nose, ears, manus and pes was restored to the normal color from the temporary cyanosis caused by bleeding and 3-4 hours were required for awakening from the anesthesia. This may indicate that the infused bovine Hb-containing hemosome was found to function as oxygen and carbon dioxide transporter. Although toxic effect of the infused hemosomes in surviving animals was not investigated in detail the effect of the hemosome on the survival in the bled rats was satisfactory, because the four of five rats bled one-third of total blood (6% of B.W.) were survival after infusion of the hemosome solution for about

Table 2. The changes in hemoglobin oxidation products in hemosome during incubation of the Hb-containing liposomes at 37°C

Content of liposome	Oxidation products	Crystallized Hb	Hemosome	
			Immediately after preparation	After 24hr-incubation at 37°C
Bovine-Hb	OxyHb	40	10	2
	MetHb	60	89	92
	Hemichrome	—	1	6
Human-Hb	OxyHb	14	5	2
	MetHb	86	95	96
	Hemichrome	—	—	2

The hemosomes were incubated at 37°C with mild shaking in Tris-buffered saline(pH 7.4) for one day and results are expressed as percent of total Hb.

6 weeks until they were sacrified and one died within 24 hours after the transfusion. Control experiments exchanging blood with buffered-saline instead of hemosome solution were not performed because it has been demonstrated previously to be lethal to the life of the bled rats (Hah et al, 1990).

DISCUSSION

Hemosome possesses the essential properties required as a red blood cell substitute and these functional properties of the encapsulated hemoglobin have been well established (Farmer and Gaber, 1987) and cross-linked hemoglobin were also suggested as blood substitutes (Snyder et al, 1987). There are several methods for the encapsulation of hemoglobin in vesicles (Szoka & Papahadjopoulos, 1978; Arakawa & Kondo, 1979; Djordjevich & Miller, 1980; Gaber et al, 1983; and Jopsik et al, 1989) and the size of hemoglobin vesicles varied from 0.01 μm to 30 μm in diameter according to the methods used for hemosome formation. Generally, the vesicles obtained from the organic or detergent methods seem to be smaller than that from the film methods. The large multilamellar or very small unilamellar lipid vesicles (liposomes) could be improved to be appropriate in size for use (Anzai et al, 1990; Bosworth et al, 1982). In the structure of artificial RBC (hemosome), unilamellar and small vesicles are thought to be more useful than multilamellar and large vesicles. And further, a reinforcement of hemoglobin vesicle is required in order to strengthen the membrane against mechanical stress in capillaries. The reason is because, in natural RBC, Saxton (1990) described that spectrin network on cytoplasmic surface of the RBC membrane is modeled upon a triangular lattice of spectin tetramers for stabilizing that RBC of diameter 8 μm passes through capillaries of diameter 2-3 μm repeatedly. On the function of artificial RBC, P_{50} was ranged 9-30 torr and Hill coefficient (n) was reported to be 2.4-2.9 (Farmer & Gaber, 1987; Jopski et al, 1989; Synder et al, 1987). In the hemosome prepared in present study, P_{50} was 28 torr and Hill coefficient was 2.63.

For the hemosome synthesized from purified hemoglobin and egg lecithin, α-Tocopherol, catalase, ascorbate and cholesterol were reported to protect against both hemoglobin oxidation and lipid peroxidation (Szebeni et al, 1984; Szebeni & Toth, 1986), and cholesterol changes enthalpy of the phase transition in the phospholipid membrane (Shin & Freed, 1989; Singer & Finegold, 1990). Refering to Benesch et al (1973), ligand binding by hemoglobin and

a change in the oxidation state of heme iron are accompanied by substantial alterations in absorption spectra at the wavelength of 560, 577 and 630nm and the visible specturm of ferrihemoglobin is pH-sensitive. In the present study, as we can see in the Table 2, the irreversible change in the oxidation state of hemoglobin in the hemosomes during incubation at 37°C was exhibited with spectral changes at the wavelengths. It was reported that most lipid vesicles used as drug carrier fail to stay in blood for more than a few hours owing to the opsonic activity of plasma components and the liposome removal by the cells of mononuclear-phagocytic system (Blume & Cevc, 1990). Therefore, stabilized and long-lived liposomes including hemosome are needed. Even though the life-span of hemosome is not clear in blood stream, from the result of the present study, we assumed that hemosome exists for enough period in the circulation system of the infused rat as oxygen carrier substitute until new erythrocytes are produced, because the infused hemosome solution extended the life of the drastically bled rats. Hunt et al (1985) reported that microencapsulated hemoglobin revealed damages in the kidney, liver, spleen of the infused rats. Thus, histopathological studies on the disorders which will be caused by hemosomes during circulation as blood substitute also seem to be investigated further for producing a good surrogate of blood.

REFERENCES

Anzai K, Yoshida M & Kirino Y (1990) Change in intravesicular volume of liposomes by freeze-thaw treatment as studied by ESR stopped-flow technique. *Biochim Biophys Acta* **1021**, 21-26.

Arakawa M & Kondo T (1979) Preparation and properties of poly (N^{α}, N^{ϵ}-L-lysinediylterephthaloyl) microcapsules containing hemolysate in the nanometer range. *Can J Physiol Pharmacol* **58**, 183-187

Benesch RE, Benesch R & Yung S (1973) Equations for the spectrometric analysis of hemoglobin mixtures. *Anal Biochem* **55**, 245-248

Blume G & Cevc G (1990) Liposomes for the sustained drug release in vivo. *Biochim Biophys Acta* **1029**, 91-97.

Bosworth ME, Hunt CA & Pratt D (1982) Liposome dialysis for improved size distribution. *J Pharm Sci* **71**, 806-812

Christensen CW, Reeves WC, Lasser TA & Schmidt DH (1988) Inadequate subendocardial oxygen delivery during perfluorocarbon perfusion in a canine model of ischemia. *Am Heart J* **115**, 30-37

Courtice FC (1943) The blood volume of normal animal. *J Physiol* **102**, 290-298.

Djordjevich L & Miller IF (1980) Synthetic erythrocytes from lipid encapsulated hemoglobin. *Exp Hemat* **8**, 584-592

Farmer MC & Gaber BP (1987) Lipsome-encapsulated hemoglobin as an artifical oxygen carrying system. *Methods Engymol* **149**, 185-200.

Gaber BP, Yager P, Sheridan JP & Chang EL (1983) Encapsulation of hemoglobin in phospholipid vesicles. *FEBS Lett* **153**, 285-288

Hah JS, Cho EH & Kim KJ (1990) Synthesis and use of artificial red cells. *Kor J Physiol* **24**, 15-26

Hunt CA, Burnette RR, MacGregor RD & Strubbe AE (1985) Synthesis and evaluation of a prototypal artificial red cell. *Science* **230**, 1165-1168

Jopski B, Pirkl V, Jaroni H-W, Schubert R & Schmidt K-H (1989) Preparation of hemoglobin-containing liposomes using octylglucoside and octyltetraoxyethylene. *Biochim Biophys Acta* **978**, 79-84

Lieberthal W, Wolf EF, Merrill EW, Levinsky LG & Voleri CR (1987) Hemodynamic effects of different preparations of stroma free hemolysates in the isolated perfused rat kidney. *Life Sci* **41**, 2525-2533

Lutz J & Stark M (1987) Half life and changes in the composition of a perfluorochemical emulsion within the vascular system of rats. *Pflugers Arch* **410**, 181-184

Ozaki N, Tokunaga Y, Ikai I, Zaina M, Sakai Y, Shimahara, Y, Kamiyama Y, Yamaoka Y, Ozawa & Nakase Y (1989) Pyridine nucleotide fluorometry in preserved porcine liver with fluorocarbon emulsion. *Transplantation* **48**,

198-201

Saxton MJ (1990) The membrane skeleton of erythrocyte. *Biophys J* **57**, 1167-1177

Shin Y-K & Freed JH (1989) Thermodynamics of phosphatidylcholine-cholesterol mixed model membranes in the liquid crystalline state studied by the orientational order parameter. *Biophys J* **56**, 1093-1100

Singer MA & Finegold L (1990) Cholesterol interacts with all of the lipid in bilayer membranes. *Biophys J* **57**, 153-156

Snyder SR, Welty EV, Walder RY, Williams LA & Walder JA (1987) HbXL 99α: A hemoglobin derivative that is cross-linked between the α-subunits is useful as a blood substitute. *Proc Natl Acad Sci* **84**, 7280-7284

Szebeni J, Winterbourn CC & Carrell W (1984) Oxidative interactions between haemoglobin and membrane lipid. *Biochem J* **229**, 685-692.

Szebeni J, Iorio EEDi, Hauser H & Winterhalter KH (1985) Encapsulation of hemoglobin at phospholipid liposomes: Characterization and stability. *Biochemistry* **24**, 2827-2832.

Szebeni J & Toth K (1986) Lipid peroxidation in hemoglobin-containing lipsomes. *Biochim Biophys Acta* **857**, 139-145

Szoka F & Paphadjopoulos D (1978) Procedure for preparation of liposomes with large internal aqueous space and capture by reverse-phase evaporation. *Proc Natl Acad Sci* **75**, 4184-4198

Tuliani VV, O'Rear EA, Fung BM & Sierra BD (1988) Interaction between erythrocytes and a perfluoro-chemical blood substitute. *J Biomed Mat Res* **22**, 45-61

van der Plas J, Rossen A de V-V, Koorevaar JJ, Buursma A, Zijlstra WG & Bakker JC (1988) Purification and physical characteristics of a hemoglobin solution modified by coupling to 2-nor-2-formylpyridoxal 5'-phosphate (NFPLP). *Transfusion* **28**, 525-530.