by adding 100 μl of 1 N H_2SO_4, and the absorbance was measured at 490 nm using a Microplate reader (model 680; Bio-Rad Laboratories, Tokyo, Japan).

Quantitative Determination of Anti-Lipid IgM. A 10-nmol aliquot of each lipid (DPPC, cholesterol, DHSG, or PEG-DSPE) in 50 μl of 100% ethanol was added to 96-well plates (Immuno 96 MicroWell Plate; Nalge Nunc International). The plates were incubated for 4 h at 37°C to dry completely. After incubation, blocking solution was added to each well, and the plate was incubated for 2 h at 25°C. Following processes were identical to those described under *Quantitative Determination of Anti-HbV IgG and IgM*.

Data Analysis. Pharmacokinetic analyses, after HbV injections, involved the use of a two-compartment model, and pharmacokinetic parameters were estimated by curve fitting. Pharmacokinetic parameters were calculated by fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). The uptake clearance (CL_{uptake}) was calculated as described in a previous report using integration plot analysis at designated times (from 1–30 min), during which time the efflux and/or elimination of radioactivity from tissues were negligible (Murata et al., 1998). Data are shown as mean ± S.D. for the indicated number of animals. The Bonferroni test was used for comparisons with a saline injection group within each group. Significant differences among each group were examined using the Student's t test. A probability value of $p < 0.05$ was considered to indicate statistical significance.

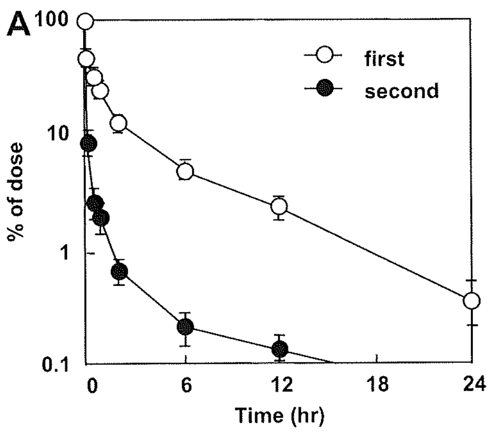
Results

Pharmacokinetic Properties of HbV after Repeated Injection of 0.1 or 1400 mg Hb/kg. The fate of the ^{125}I-HbV administered to mice was evaluated by determining the residual trichloroacetic acid-precipitable radioactivity in plasma. In this study, the time interval for injection was selected for 7 days based on the previous report, in which ABC phenomenon in mice was observed the most strongly when the time interval for the injection was 7 to 10 days (Ishida et al., 2003b). In addition, blood viscosity after high-dose administration of HbV was equal to that before administration of HbV (Sakai et al., 1998), and repeated infusion of HbV had no adverse clinical signs or symptoms (Sakai et al., 2004a). Figure 1 shows the time course for the plasma concentration curve for ^{125}I-HbV administered once or twice to mice, and Table 1 lists the pharmacokinetic parameters obtained using the two-compartment model.

At a dose of 0.1 mg Hb/kg, plasma HbV in the second injection was rapidly cleared compared with that in the first injection (Fig. 1A). The half-life ($t_{1/2}$) in the second injection was reduced significantly—by approximately half—compared with that in the first injection. Accompanied by the reduction in $t_{1/2}$, the area under the concentration-time curve (AUC) was also significantly decreased (27.1 ± 18 and 4.5 ± 3.8 h*% of dose/ml, $p < 0.001$, for first and second injection, respectively), whereas plasma clearance (CL) was significantly increased in the second injection compared with that in the first injection (3.69 ± 0.4 and 22.3 ± 8.1 ml/h, $p < 0.001$, for the first and second injections, respectively). However, the distribution volume of the central compartment (V_1) remained unchanged as the result of repeated injections (Table 1).

At a dose of 1400 mg Hb/kg, the values of $t_{1/2}$ and CL in the second injection were not significantly different from those for the first injection, but the AUC was decreased slightly, in the case of the second injection (829 ± 38 and 695 ± 38 h*% of dose/ml, $p < 0.05$, for first and second injections, respectively) (Table 1).

Effect of Repeated Injection on the Hepatic and Splenic Distribution of HbV. Because liver is the major distribution organ for HbV (Taguchi et al., 2009b), the effect of repeated injections on the hepatic distribution of HbV was examined. Figure 2 shows the time course distribution for ^{125}I-HbV (percentage of injection of dose) in the liver after the administration of ^{125}I-HbV once or twice. Up to 0.5 h after the injection of ^{125}I-HbV at a dose of 0.1 mg Hb/kg, the hepatic distribution of ^{125}I-HbV in the second injection was much higher than that in the first injection (Fig. 2A, inset). However, after 0.5 h or more,



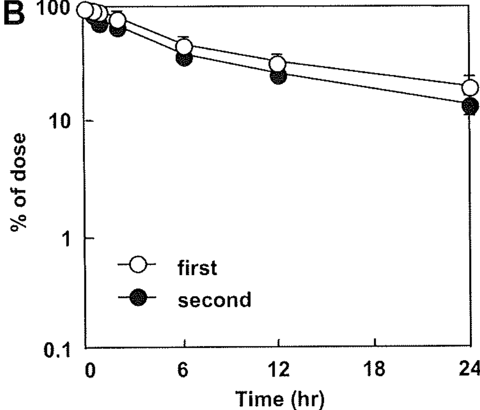


FIG. 1. Plasma concentration curve of ^{125}I-HbV after the first injection (open symbol) or the second injection (filled symbol) of ^{125}I-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of a nonlabeled HbV suspension or ^{125}I-HbV to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received the ^{125}I-HbV suspension to the tail vein. Blood was collected from the inferior vena cava under ether anesthesia, and plasma was obtained. Each point represents the mean ± S.D. (n = 3–6).

the differences between the first and second injections were minor (Fig. 2A). From the beginning after an HbV injection at a dose of 1400 mg Hb/kg, hepatic distributions of ^{125}I-HbV were similar between the first and the second injection (Fig. 2B, inset), and this tendency was maintained for periods of up to 72 h.

We next calculated the CL_{uptake} in the liver (Table 2). At a dose of 0.1 mg Hb/kg, the CL_{uptake} for the second injection was 8.5 times higher than that for the first injection (3.5 ± 0.4 and 29.6 ± 18 ml/h, $p < 0.01$, for the first and the second injection, respectively), whereas at a dosage of 1400 mg Hb/kg, the CL_{uptake} for the second injection was only 1.5 times higher than that for the first injection (0.26 ± 0.04 and 0.37 ± 0.03 ml/h, $p < 0.05$, for the first and the second injection, respectively).

Because the spleen is an another major distribution organ of HbV (Taguchi et al., 2009b) and an essential organ in terms of inducing the ABC phenomenon (Ishida et al., 2006a), we also examined the time course for the distribution of ^{125}I-HbV (percentage of injection of dose) in the spleen. For periods up to 1 h after HbV injection, the splenic distributions of ^{125}I-HbV in the first and the second injections were not greatly different for doses of both 0.1 and 1400 mg Hb/kg (Fig. 3, A and B, insert). However, 1 h or more after the second injection, higher splenic distributions of HbV were observed in both the low- and high-dose groups compared with those in the first injection (Fig. 3, A and B). In addition, we calculated the CL_{uptake} in

TABLE 1

Pharmacokinetic parameters for HbV after one or two injections of ^{125}I-HbV in mice

Mice received a single or double injection of ^{125}I-HbV (0.1 and 1400 mg Hb/kg) containing 5% rHSA. At each time after the ^{125}I-HbV injection, blood was collected from the inferior vena cava, and plasma was obtained. Each parameter was calculated by MULTI using the two-compartment model. The values are mean ± S.D. (n = 3–6).

	0.1 mg Hb/kg		1400 mg Hb/kg	
	First Injection	Second Injection	First Injection	Second Injection
$t_{1/2}$ (h)	2.7 ± 0.2	1.3 ± 0.3*	18.8 ± 1.3	17.4 ± 3.9
AUC (h*% of dose/ml)	27.1 ± 18	4.5 ± 3.8**	829 ± 38	695 ± 38*
CL (ml/h)	3.69 ± 0.4	22.3 ± 8.1**	0.12 ± 0.04	0.14 ± 0.05
V_1 (ml)	3.1 ± 0.3	3.2 ± 0.3	1.75 ± 0.6	1.81 ± 0.3

V_1, the distribution volume of the central compartment.
* $p < 0.05$, ** $p < 0.001$ vs. first injection.

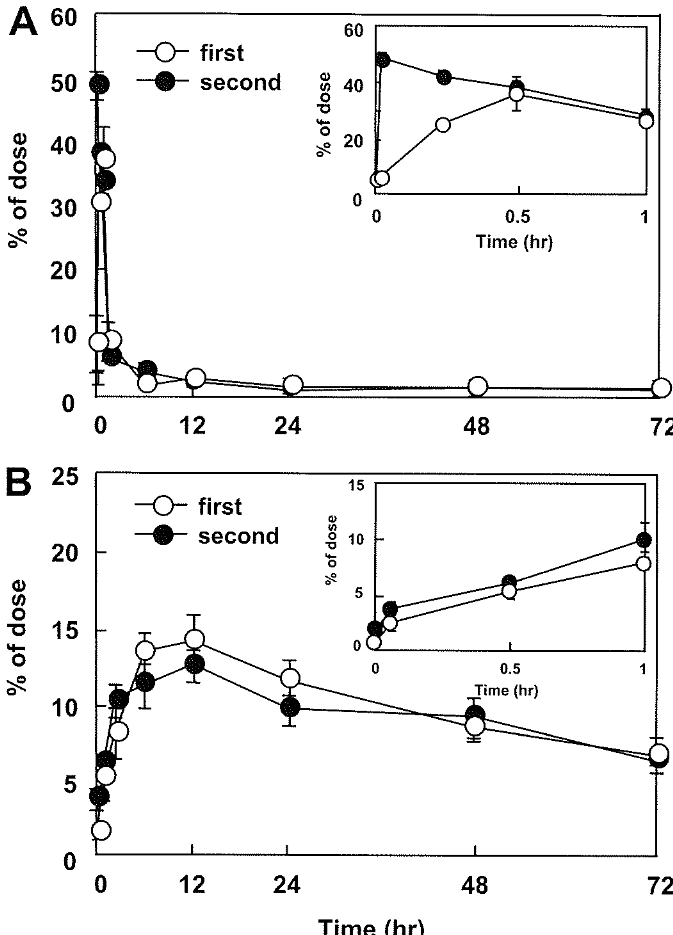


FIG. 2. Time courses for radioactivity in liver after the first injection (open circles) or the second injection (filled circles) of ^{125}I-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of nonlabeled HbV suspension or ^{125}I-HbV to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received the ^{125}I-HbV suspension to the tail vein. Each point represents the mean ± S.D. (n = 3–6).

the spleen. At a dose of 0.1 mg Hb/kg, the CL_{uptake} for the second injection was 4.5 times higher than that for the first injection (1.6 ± 0.1 and 7.2 ± 3.2 ml/h, $p < 0.01$, for the first and the second injection, respectively). At a dose of 1400 mg Hb/kg, the CL_{uptake} for the second injection (0.07 ± 0.02 ml/h) was not significantly changed compared with that for the first injection (0.05 ± 0.01 ml/h).

Furthermore, we also examined the distribution of ^{125}I-HbV in the kidney, lung, and heart at doses of both 0.1 and 1400 mg Hb/kg. No significant differences were observed between the first and the second injections (data not shown).

Determination of IgG and IgM against HbV after HbV Injection. In a previous study, it was reported that IgM, which is produced by the preinjection of PEGylated liposomes, is strongly involved in the induction of the ABC phenomenon (Ishida et al., 2006b). Therefore, we examined the issue of whether IgG or IgM against HbV is elicited by an initial injection of saline or HbV at a dose of 0.1 and 1400 mg Hb/kg. Figure 4 shows the quantitative determination of plasma IgG (A) and IgM (B) against HbV. Negligible levels of IgG were elicited against HbV in all the injection groups at 3, 7, and 10 days after the injection of saline or HbV (Fig. 4A). In contrast, the IgM against HbV was significantly elicited starting from 3 days after the first injection of HbV at a dose of 0.1 mg Hb/kg (Fig. 4B). On the other hand, at a dose of 1400 mg Hb/kg, the IgM against HbV was significantly elicited starting from 7 days after the first injection. At 10 days after the first injection, IgM levels against HbV at a dose of 1400 mg Hb/kg were significantly higher than the levels at a dose of 0.1 mg Hb/kg ($p < 0.01$) (Fig. 4B).

Determination of the Specific Recognition Site of IgM against HbV. To evaluate the specific recognition site of IgM against HbV, a modified ELISA was employed using each lipid component of HbV. Figure 5 shows data for the quantitative determination of the specific recognition site of IgM against HbV at 3, 7, and 10 days after the first injection of HbV at doses of 0.1 or 1400 mg Hb/kg. At a dose of 0.1 mg Hb/kg, strong binding of IgM to DSPE-PEG was observed, starting at day 3 after the first injection, whereas a dramatic enhancement in the binding of IgM to DSPE-PEG was observed, starting at 7 days at a dose of 1400 mg Hb/kg. On the other hand, IgM against other lipid components (DPPC, cholesterol, and DHSG) were negligible during all the times examined after the injection of both low and high doses of HbV.

Discussion

As discussed in the introduction, HbV is a red blood cell substitute, the proposed dose of which is 1400 mg Hb/kg. This dosage is more than 100 times higher than that of liposome preparations used as pharmaceuticals, and the use of multiple doses is planned under clinical situations. Therefore, an investigation of whether repeated HbV injections induce the ABC phenomenon is a necessity. However, little information is available on the ABC phenomenon at such extraordinarily high doses of liposomes. In this study, we found an interesting phenomenon, namely, that repeated injections of HbV to mice at a dose of 1400 mg Hb/kg did not seem to induce the ABC phenomenon, even though the plasma levels of IgM against HbV were significantly elevated.

When mice received injections of a low-dose (0.1 mg Hb/kg) HbV, a dose that Ishida et al. (2003b) reported induced the ABC phenom-

TABLE 2

Uptake clearance of HbV in the liver and spleen of mice receiving injections of ^{125}I-HbV

All of the mice received a single or double injection of ^{125}I-HbV (0.1 and 1400 mg Hb/kg) containing 5% rHSA. The uptake clearance for each organ was calculated by integration plot analysis at designated times from 1 to 30 min after injection. The values are mean ± S.D. (n = 3–6).

	0.1 mg Hb/kg		1400 mg Hb/kg	
	First Injection	Second Injection	First Injection	Second Injection
Liver (ml/h)	3.5 ± 0.4	29.6 ± 18*	0.26 ± 0.04	0.37 ± 0.03**
Spleen (ml/h)	1.6 ± 0.1	7.2 ± 3.2*	0.05 ± 0.01	0.07 ± 0.02

* $p < 0.01$ and ** $p < 0.05$ vs. first injection.

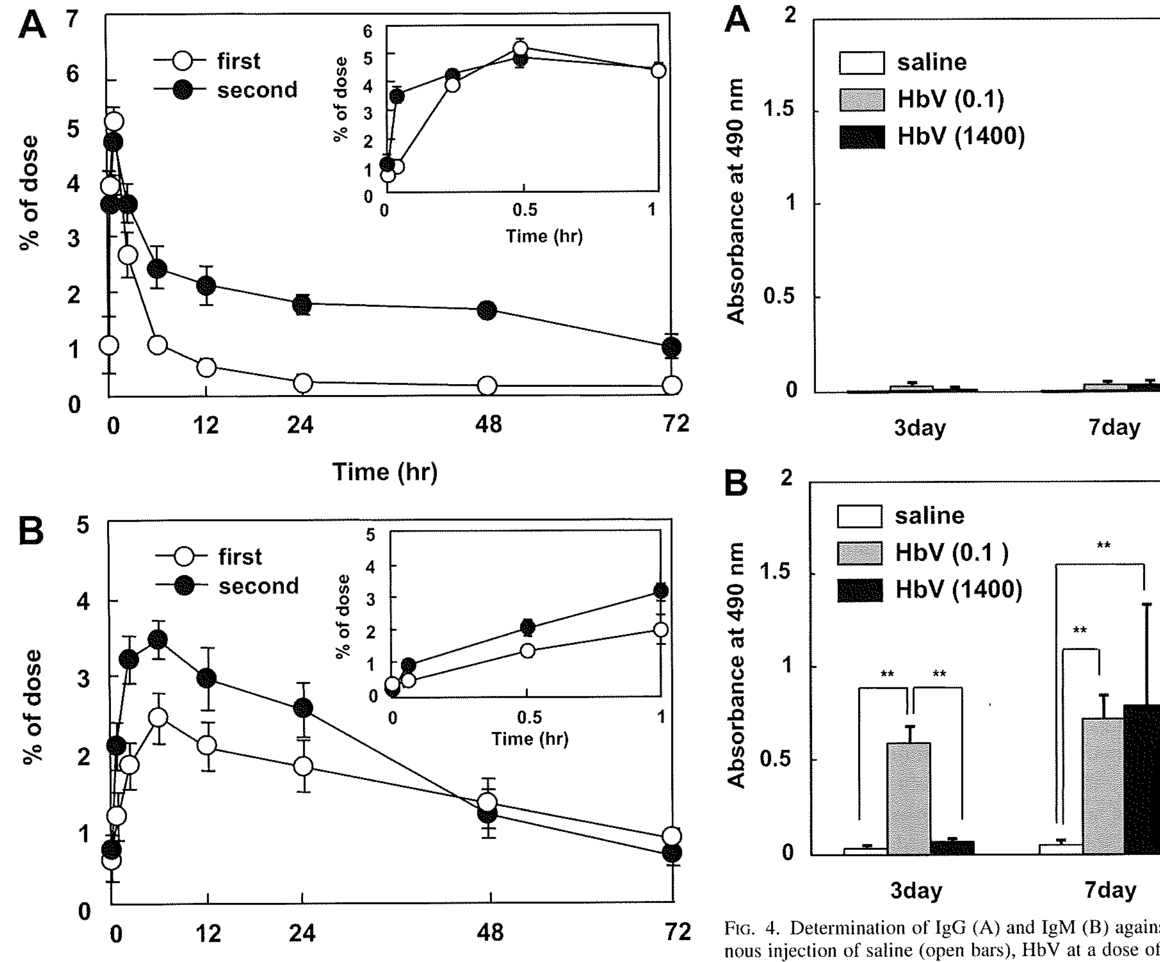


FIG. 3. Time courses for radioactivity in spleen after the first injection (open circles) or the second injection (filled circles) of ^{125}H-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of nonlabeled HbV suspension or ^{125}I-HbV to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received the ^{125}I-HbV suspension to the tail vein. Each point represents the mean ± S.D. (n = 3–6).

FIG. 4. Determination of IgG (A) and IgM (B) against HbV after a single intravenous injection of saline (open bars), HbV at a dose of 0.1 mg Hb/kg (gray bars) or 1400 mg Hb/kg (closed bars) in mice. The ddY mice received injections of saline or HbV (0.1 or 1400 mg Hb/kg) to the tail vein. At 3, 7, and 10 days after injection of saline or HbV, blood was collected from the inferior vena cava, and plasma was obtained. Anti-HbV IgG and IgM were detected with ELISA. Each bar represents the mean ± S.D. (n = 4). **, $p < 0.01$.

enon, the ABC phenomenon was clearly induced at 7 days postinjection (Fig. 1A; Table 1). Consequently, the pharmacokinetics of HbV was markedly changed. For example, the $t_{1/2}$ and AUC for HbV in the second injection were significantly decreased compared with the values for the first injection, and the CL for the second injection was significantly increased. In addition, the hepatic distribution of ^{125}I-HbV after the second injection at a dose of 0.1 mg Hb/kg was increased for periods of up to 30 min (Fig. 2A) with an increase in hepatic uptake clearance for the second injection (Table 2). In a previous study, Dams et al. (2000) reported that, in mice that were administered liposomes at weekly intervals at a dose of 5 μmol of phospholipids/kg, the ABC phenomenon was not induced. It is well known that a variety of factors, including the lipid dose and physicochemical properties (degree of PEGylation, PEG chain length, surface charge and size) of the initially injected liposome, strongly affect the pharmacokinetic response to subsequent injection (Ishida et al., 2004). For example, it appears that the ABC phenomenon was not caused by preinjection with smaller-sized polymeric micelles but was triggered by preinjection with larger-sized polymeric micelles (Koide et al., 2008). Wang et al. (2005) found that the induction and magnitude of the ABC phenomenon were also influenced by the lipid composition.

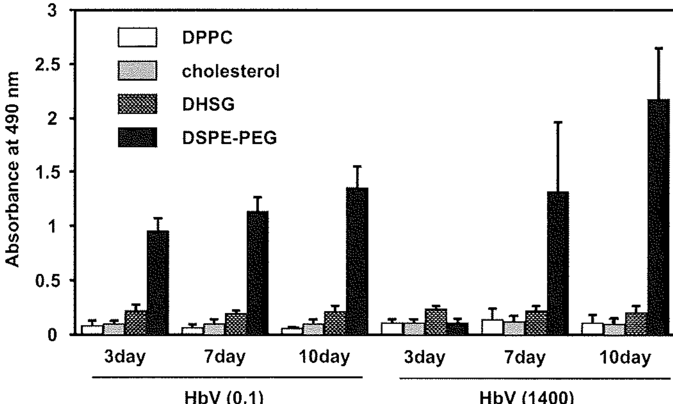


FIG. 5. Determination of the specific recognition site of IgM against HbV. A 10-nmol sample of DPPC (open bar), cholesterol (gray bar), DHSG (hatched bar), or DSPE-PEG (closed bar) in 50 μl of 100% ethanol was placed in a dish to completely remove the ethanol. IgM against each lipid component was detected by ELISA. Each point represents the mean ± S.D. (n = 4).

Therefore, such contradictory findings between the present and Dams' study could be the result of differences in the physicochemical properties of the liposomes used in the study, such as particle size or lipid composition. In fact, the diameter of the HbV liposomes used was approximately 250 nm, whereas the diameter of the PEGylated liposome used by Dams et al. (2000) was approximately 80 nm. In addition, our HbV was composed of DPPC, cholesterol, DPSH, and DSPE-PEG at a molar ratio of 5:5:1:0.3, whereas the PEGylated liposome used by Dams et al. (2000) was composed of partially hydrogenated egg-phosphatidylcholine, cholesterol, *N*-hydroxysuccinimidyl hydrazino nicotinate hydrochloride DSPE, and DSPE-PEG at a molar ratio of 1.85:1:0.07:0.15.

In addition, Ishida et al. (2006b) reported that the ABC phenomenon was induced by the selective binding of IgM to the second injected PEGylated liposome, and subsequent complement activation by IgM resulted in an accelerated clearance and enhanced hepatic uptake of the second injected PEGylated liposome. Wang et al. (2007) also reported that the hepatic clearance of liposomes was positively correlated with the plasma levels of IgM against the PEGylated liposome. In this study, we also found a substantial elevation in plasma IgM levels at both low (0.1 mg Hb/kg) and high doses (1400 mg Hb/kg contains more than 100 μmol of phospholipids/kg). Because the degree of IgM elevation at a high dose of HbV was significantly larger than that for a low dose, the IgM response against HbV might be dose-dependent.

To identify the recognition site of anti-HbV IgM, we also examined the production of IgM in the presence of each of the lipid components of HbV. Among these components, only anti-HbV IgM was found to react with DSPE-PEG (Fig. 5). In addition, the IgM level against HbV was well correlated with that against DSPE-PEG (data not shown, r = 0.611, $p < 0.01$). These data clearly indicate that the recognition site of anti-HbV IgM is mainly DSPE-PEG. It is also noteworthy that the synthetic negatively charged lipid (DHSG) in HbV does not seem to show a remarkable immunogenicity. It was previously reported that the spleen, especially the marginal zone, plays an important role in the induction of the ABC phenomenon, which elicits IgM against PEG, and the IgM responded in a T cell-independent manner in rats and mice (Ishida et al., 2006a, 2007). The fact that plasma IgM levels against HbV and DSPE-PEG were significantly enhanced at both doses (Figs. 4 and 5) suggests that HbV interacts directly with the marginal zone. Future studies will be needed to elucidate the details of the mechanism of the elicitation of IgM against HbV.

In contrast to the low-dose treatment, the injection of a high dose (1400 mg Hb/kg) of HbV did not appear to induce the ABC phenomenon in mice (Fig. 1B; Table 1). In fact, the hepatic uptake clearance for the second injection was only 1.5 times higher than that for the first injection (Table 2). Ishida et al. (2004) previously reported that the lipid dose of a prior injection of liposomes strongly affected the pharmacokinetic behavior of a subsequent injection at a dose of from 0.001 to 25 μmol of phospholipids/kg. They reported that liver accumulation in mice increased sigmoidally with increasing lipid dose, whereas the blood concentration sigmoidally decreased with increasing lipid dose. In general, the increased hepatic or splenic distributions of liposomes were accompanied by an increased scavenging of liposome by MPS, such as Kupffer cells and red pulp zone splenocytes (Goins et al., 1995). MPS or any other systems that are involved in the removal of liposomes are influenced by the injection dose (Laverman et al., 2000), and the uptake by MPS was saturated with increasing doses of liposomes. Our previous study showed that the distribution of HbV in the liver was saturated at 1400 mg Hb/kg (more than 100 μmol of phospholipids/kg) but not at 200 mg Hb/kg (approximately 25 μmol of phospholipids/kg) (Taguchi et al., 2009b). Consequently, HbV at a dose of 1400 mg Hb/kg did not appear to induce the ABC phenomenon, even though accompanied with remarkable IgM elicitation, because the hepatic uptake of HbV via MPS was saturated in the case of a high-dose injection. From these results, it was expected that ABC phenomenon might not be apparently induced at various intervals at proposed dose of HbV (1400 mg Hb/kg) because ABC phenomenon in mice was observed the most strongly at the 7- to 10-day interval (Ishida et al., 2003b). In fact, we previously reported that ABC phenomenon was not induced in hemorrhagic shock model rat, when HbV was injected at a dose of 1400 mg Hb/kg at hourly intervals (Taguchi et al., 2009a), at which the patients with massive hemorrhage are transfused.

However, our study has limitations with respect to explanation of full-length study of the ABC phenomenon of HbV. We have not examined the plasma IgM levels when multiple high doses of HbVs were administered. Dams et al. (2000) previously reported that weekly injections of *N*-hydroxysuccinimidyl hydrazino nicotinate hydrochloride PEG liposomes dramatically influenced the circulatory half-life at second injection, but the effect was almost normalized at fourth injection. Therefore, it seems that the higher levels of IgM elevations are not observed after multiple high-dose administration of HbV. On this point, further study could be needed for elucidating the effect of multiple high-dose administration of HbV on their pharmacokinetics.

In conclusion, the present study clearly shows that repeated injections of HbV induce the ABC phenomenon, when the first injection of HbV was a dose of 0.1 mg Hb/kg, but was not apparent at a dose of 1400 mg Hb/kg. These results suggest that, in a clinical situation, the repeated use of HbV at a dose of 1400 mg Hb/kg would not be expected to induce the ABC phenomenon. Thus, it is unlikely to be necessary to consider the ABC phenomenon in an administration schedule or regimen of HbV treatment as a red blood cell substitute.

References

Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, Corstens FH, and Boerman OC (2000) Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J Pharmacol Exp Ther* **292:**1071–1079.

Goins B, Klipper R, Sanders J, Cliff RO, Rudolph AS, and Phillips WT (1995) Physiological responses, organ distribution, and circulation kinetics in anesthetized rats after hypovolemic exchange transfusion with technetium-99m-labeled liposome-encapsulated hemoglobin. *Shock* **4:**121–130.

Ishida T, Ichihara M, Wang X, and Kiwada H (2006a) Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes. *J Control Release* **115:** 243–250.

Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, and Kiwada H (2006b) Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. *J Control Release* **112:**15–25.

Ishida T, Ichikawa T, Ichihara M, Sadzuka Y, and Kiwada H (2004) Effect of the physicochemical properties of initially injected liposomes on the clearance of subsequently injected PEGylated liposomes in mice. *J Control Release* **95**:403–412.

Ishida T, Maeda R, Ichihara M, Irimura K, and Kiwada H (2003a) Accelerated clearance of PEGylated liposomes in rats after repeated injections. *J Control Release* **88**:35–42.

Ishida T, Masuda K, Ichikawa T, Ichihara M, Irimura K, and Kiwada H (2003b) Accelerated clearance of a second injection of PEGylated liposomes in mice. *Int J Pharm* **255**:167–174.

Ishida T, Wang X, Shimizu T, Nawata K, and Kiwada H (2007) PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *J Control Release* **122**:349–355.

Koide H, Asai T, Hatanaka K, Urakami T, Ishii T, Kenjo E, Nishihara M, Yokoyama M, Ishida T, Kiwada H, et al. (2008) Particle size-dependent triggering of accelerated blood clearance phenomenon. *Int J Pharm* **362**:197–200.

Laverman P, Brouwers AH, Dams ET, Oyen WJ, Storm G, van Rooijen N, Corstens FH, and Boerman OC (2000) Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. *J Pharmacol Exp Ther* **293**:996–1001.

Lee J, Lee J, Yoon S, and Nho K (2006) Pharmacokinetics of 125I-radiolabelled PEG-hemoglobin SB1. *Artif Cells Blood Substit Immobil Biotechnol* **34**:277–292.

Murata M, Tamai I, Sai Y, Nagata O, Kato H, Sugiyama Y, and Tsuji A (1998) Hepatobiliary transport kinetics of HSR-903, a new quinolone antibacterial agent. *Drug Metab Dispos* **26**:1113–1119.

Noble CO, Krauze MT, Drummond DC, Yamashita Y, Saito R, Berger MS, Kirpotin DB, Bankiewicz KS, and Park JW (2006) Novel nanoliposomal CPT-11 infused by convection-enhanced delivery in intracranial tumors: pharmacology and efficacy. *Cancer Res* **66**:2801–2806.

Okamura Y, Takeoka S, Eto K, Maekawa I, Fujie T, Maruyama H, Ikeda Y, and Handa M (2009) Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. *J Thromb Haemost* **7**:470–477.

Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* **159**:1079–1088.

Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Takaori M, Kobayashi K, and Tsuchida E (2004a) Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* **311**:874–884.

Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E (2004b) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* **32**:539–545.

Sakai H, Masada Y, Takeoka S, and Tsuchida E (2002) Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. *J Biochem* **131**:611–617.

Sakai H, Seishi Y, Obata Y, Takeoka S, Horinouichi H, Tsuchida E, and Kobayashi K (2009) Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days. *Shock* **31**:192–200.

Sakai H, Sou K, Horinouchi H, Kobayashi K, and Tsuchida E (2008) Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. *J Intern Med* **263**:4–15.

Sakai H, Takeoka S, Park SI, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, and Tsuchida E (1997) Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjug Chem* **8**:23–30.

Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, and Tsuchida E (2003) Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. *Clin Chem Lab Med* **41**:222–231.

Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, Tsuchida E, and Intaglietta M (1998) Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. *J Biomed Mater Res* **40**:66–78.

Sakai H and Tsuchida E (2007) Hemoglobin-vesicles for a transfusion alternative and targeted oxygen delivery. *J Liposome Res* **17**:227–235.

Samad A, Sultana Y, and Aqil M (2007) Liposomal drug delivery systems: an update review. *Curr Drug Deliv* **4**:297–305.

Sou K, Klipper R, Goins B, Tsuchida E, and Phillips WT (2005) Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. *J Pharmacol Exp Ther* **312**:702–709.

Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, Horinouchi H, Tsuchida E, Kai T, and Otagiri M (2009a) Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J Control Release* **136**:232–239.

Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, Kai T, et al. (2009b) Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. *Drug Metab Dispos* **37**:1456–1463.

Tuffin G, Waelti E, Huwyler J, Hammer C, and Marti HP (2005) Immunoliposome targeting to mesangial cells: a promising strategy for specific drug delivery to the kidney. *J Am Soc Nephrol* **16**:3295–3305.

Veronese FM and Pasut G (2005) PEGylation, successful approach to drug delivery. *Drug Discov Today* **10**:1451–1458.

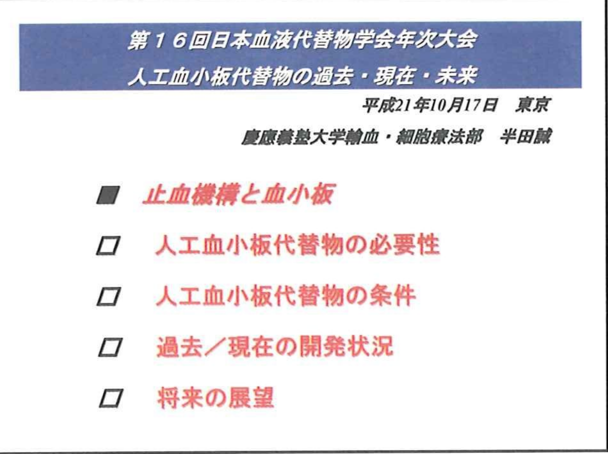
Wang X, Ishida T, and Kiwada H (2007) Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *J Control Release* **119**:236–244.

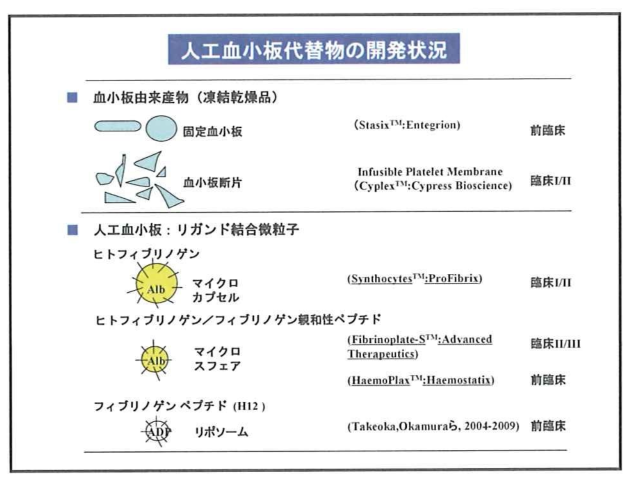
Wang XY, Ishida T, Ichihara M, and Kiwada H (2005) Influence of the physicochemical properties of liposomes on the accelerated blood clearance phenomenon in rats. *J Control Release* **104**:91–102.

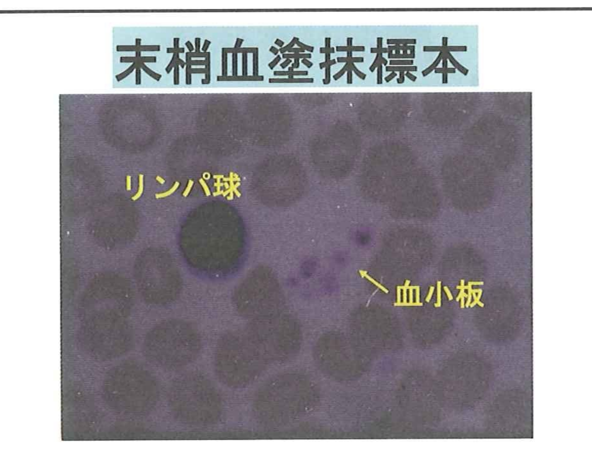
Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4**:879–885.

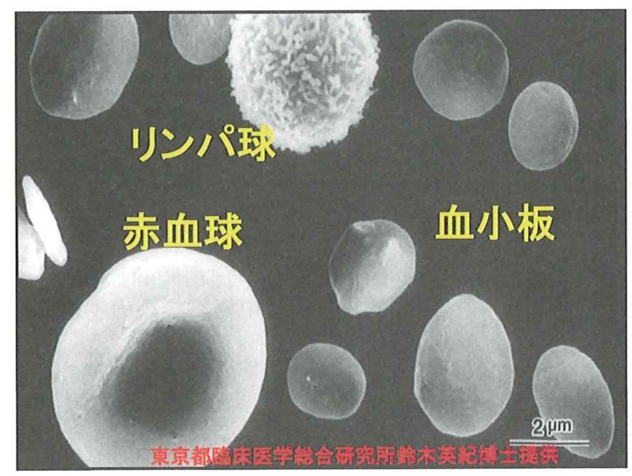
Address correspondence to: Masaki Otagiri, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: otagirim@gpo.kumamoto-u.ac.jp

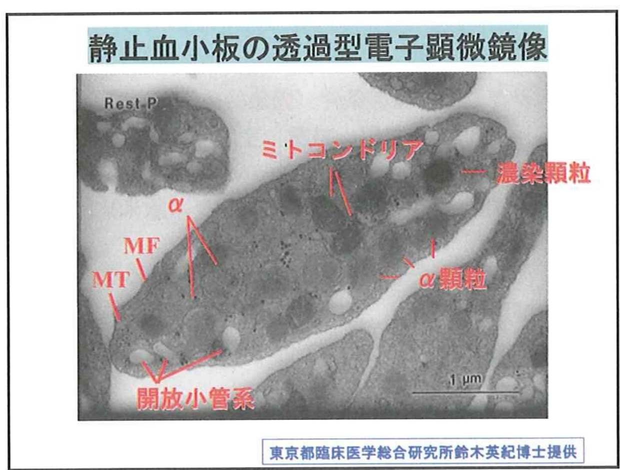
Ⅴ. その他

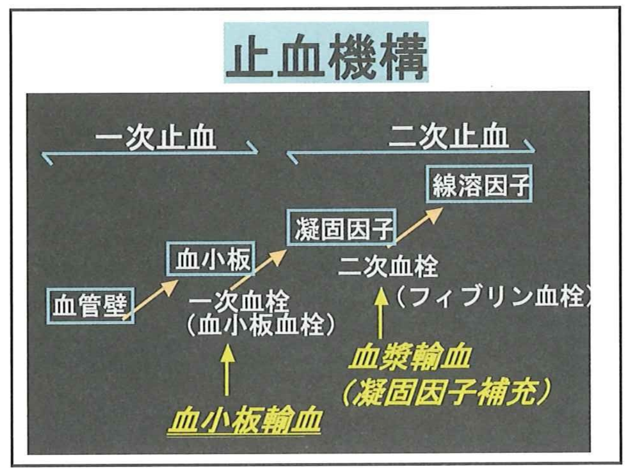

第１６回日本血液代替物学会年次大会
人工血小板代替物の過去・現在・未来
平成21年10月17日　東京
慶應義塾大学輸血・細胞療法部　半田誠
止血機構と血小板
人工血小板代替物の必要性
人工血小板代替物の条件
過去／現在の開発状況
将来の展望

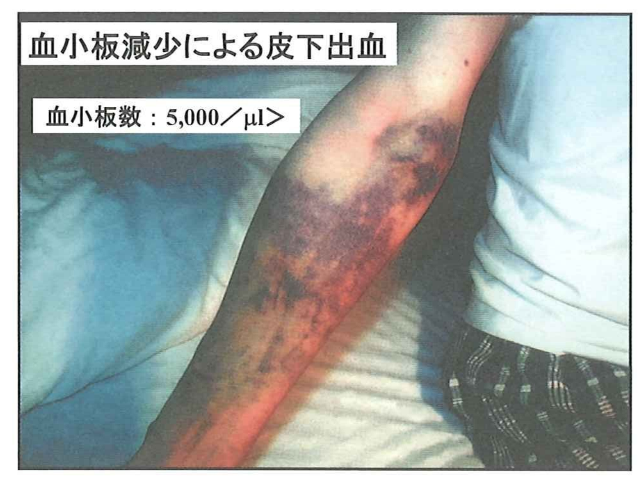

人工血小板代替物の開発状況
血小板由来産物（凍結乾燥品）
固定血小板
（StasixTM:Entegrion）
前臨床
血小板断片
Infusible Platelet Membrane
（CyplexTM:Cypress Bioscience）
臨床I/II
人工血小板：リガンド結合微粒子
ヒトフィブリノゲン
Alb
マイクロ
カプセル
(SynthocytesTM:ProFibrix)
臨床I/II
ヒトフィブリノゲン／フィブリノゲン親和性ペプチド
Alb
マイクロ
スフェア
(Fibrinoplate-STM:Advanced Therapeutics)
臨床II/III
(HaemoPlaxTM:Haemostatix)
前臨床
フィブリノゲン ペプチド (H12)
ADP
リポソーム
(Takeoka,Okamuraら, 2004-2009)
前臨床

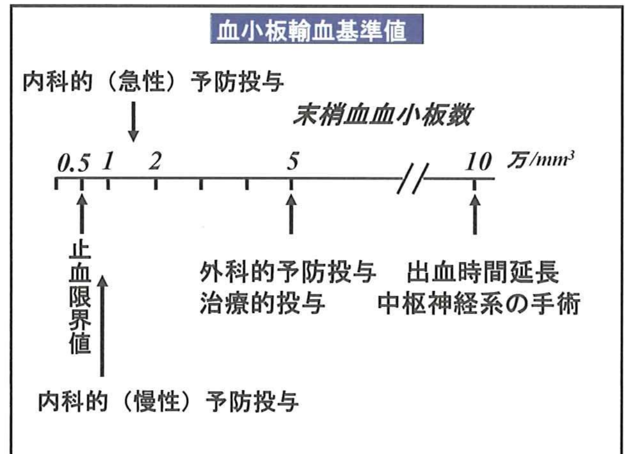

末梢血塗抹標本
リンパ球
血小板

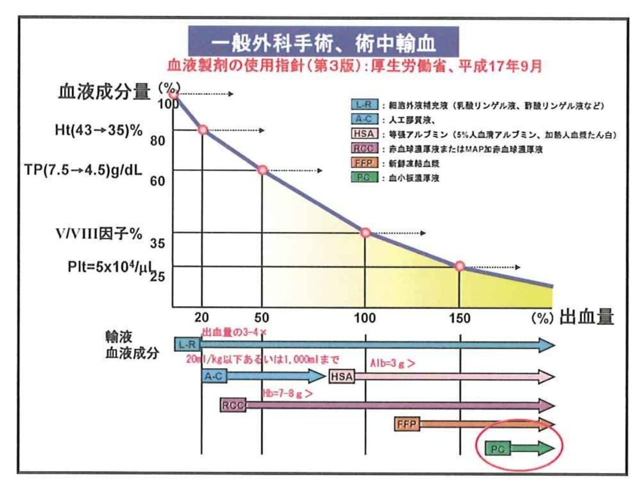

リンパ球
赤血球
血小板
2μm
東京都臨床医学総合研究所鈴木英紀博士提供


静止血小板の透過型電子顕微鏡像
Rest-P
ミトコンドリア
濃染顆粒
α
MF
MT
α顆粒
開放小管系
1μm
東京都臨床医学総合研究所鈴木英紀博士提供


止血機構
一次止血
二次止血
線溶因子
凝固因子
血小板
二次血栓
（フィブリン血栓）
血管壁
一次血栓
（血小板血栓）
血漿輸血
（凝固因子補充）
血小板輸血







大量・緊急輸血症例報告　2009年　7月8月

No.	年月日	診療科	診断名 術式・症状	年齢	性別	輸血製剤 RCC-L	FFP	PC	等張Alb	特記事項
1	2013.7.10	救急科	交通外傷(オートバイ故）オペ対応	40	M	98	40	90	11	当直時間帯 血型、ｸﾛｽﾏｯﾁ用検体同時提出
2	2013.7.11	心臓血管外	TAA	67	F	22	50	60		予定手術
3	2013.7.28	循環器内科	CPA蘇生後	62	M	30	25	10	5	当直時間帯 病棟にてPCPS使用中の出血
4	2013.8.6	心臓血管外	TAA	59	M	124	140	100	24	予定手術、事前申込RCC-LR･PC各40u オペ中追加RCC-LR110u、PC80u、
5	2013.8.9	循環器内科	CPA蘇生後	68	M	28	15	20	3	
6	2013.8.29	救急科	交通外傷(腹腔内血）オペ対応	30	M	70	40	30	8	当直時間帯、患者B型 O型RCC-LR、AB型PC･FFP使用

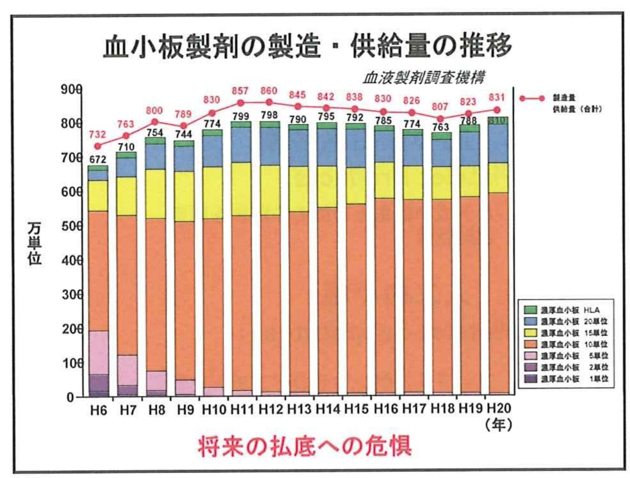
救急／救命に血小板輸血は不可欠である

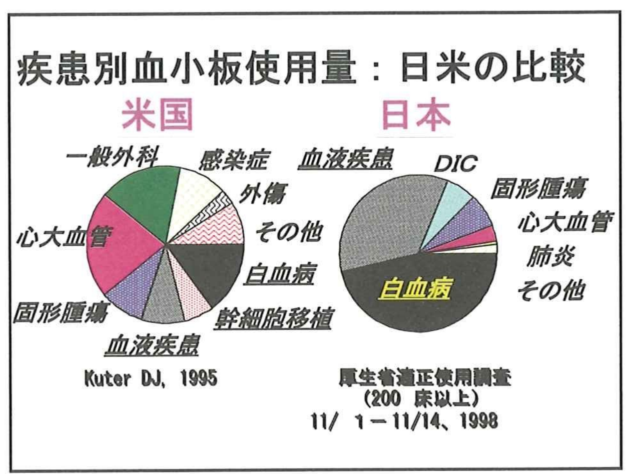
輸血用血小板製剤の問題点

1）保存期間、保存条件（緊急対応不可）
　短い：４日間、厳重：室温・振盪、予約制
2）輸血感染症
　細菌感染：要スクリーニング（欧米）
3）急性副反応
　アレルギー・アナフィラキシー反応など
4）輸血不応
　抗HLA同種抗体、抗HPA同種抗体
5）コスト
　濃厚血小板10U：￥ 76, 812
　（赤血球濃厚液2U：￥ 16, 338 ）

人工血小板代替物の過去・現在・未来

- □ *止血機構と血小板*
- ■ 人工血小板代替物の必要性
- □ 人工血小板代替物の条件
- □ 過去／現在の開発状況
- □ 将来の展望





人工血小板代替物の利点

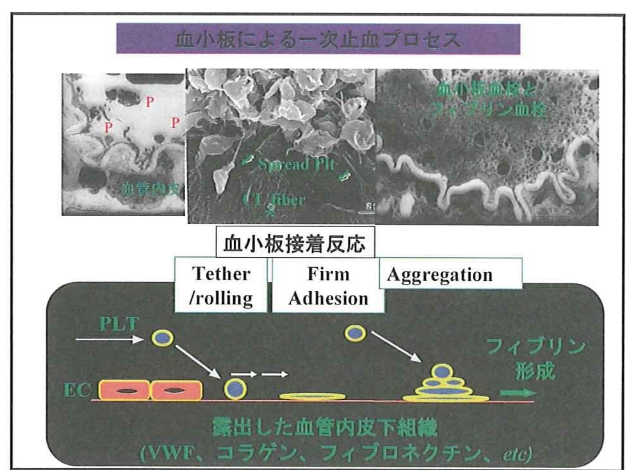
1) 緊急時の輸血対応が容易

2) 輸血副作用のリスクがない

3) 安定した血液行政が可能

人工血小板代替物の過去・現在・未来

- □ *止血機構と血小板*
- □ 人工血小板代替物の必要性
- ■ 人工血小板代替物の条件
- □ 過去／現在の開発状況
- □ 将来の展望



人工血小板代替物の条件

1) 血小板機能をすべて兼ね備える必要は無いが、止血に必要な最小限の機能を有し、残存血小板機能を補強、増幅し得る。
2) 血流中で血栓を形成しない。
3) 正常血小板の産生を抑制しない。
4) その他：長期保存可能で常時使用可能
   反復投与が可能
   副作用がない
   低コストである

人工血小板代替物の過去・現在・未来

- □ 止血機構と血小板
- □ 人工血小板代替物の必要性
- □ 人工血小板代替物の条件
- ■ 過去／現在の開発状況
- □ 将来の展望

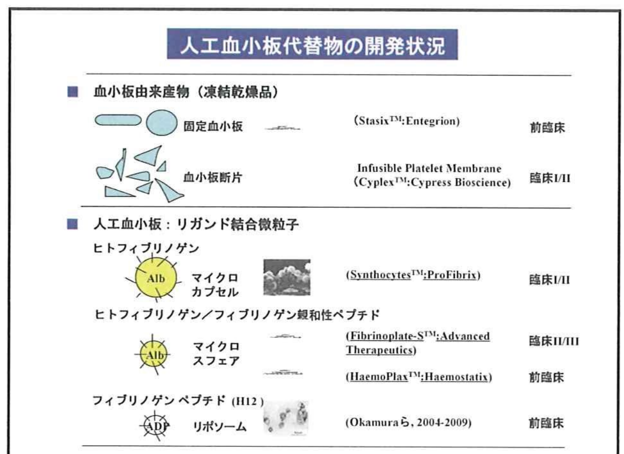
人工血小板代替物

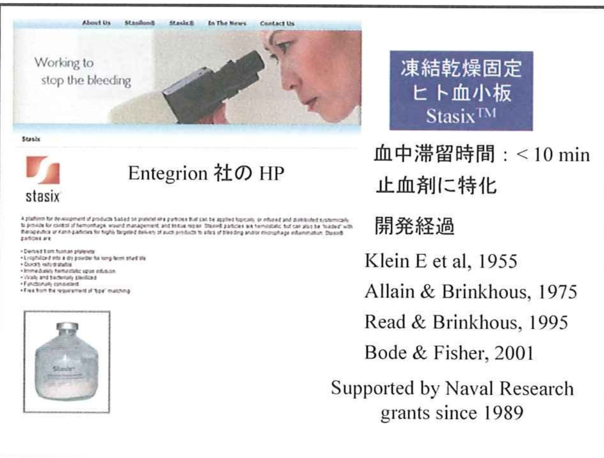
● 血小板由来産物
(Platelet Products)

ヒト血小板あるいはその断片を固定、凍結乾燥

● 人工血小板
(Platelet Substitutes)

フィブリノゲンやそのペプチドを表面固定した微粒子（アルブミン微粒子やリポソーム）





血小板代替物としてのフィブリノゲン(ペプチド)結合赤血球の効果

European Journal of Clinical Investigation (1992) **22**, 105–112

Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia

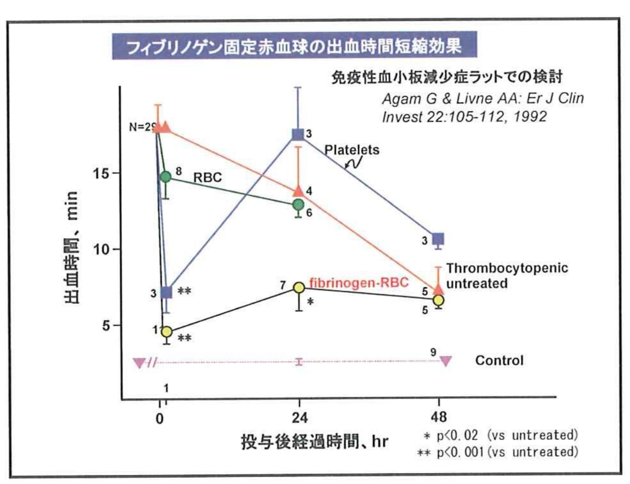
G. AGAM* & A. A. LIVNE, Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel

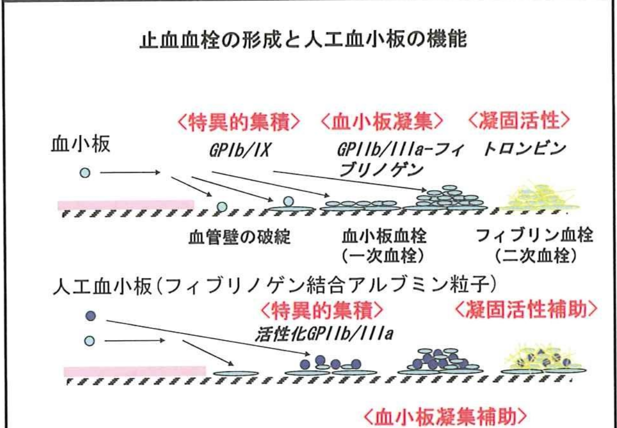
J Clin Invest 89:546-555, 1992

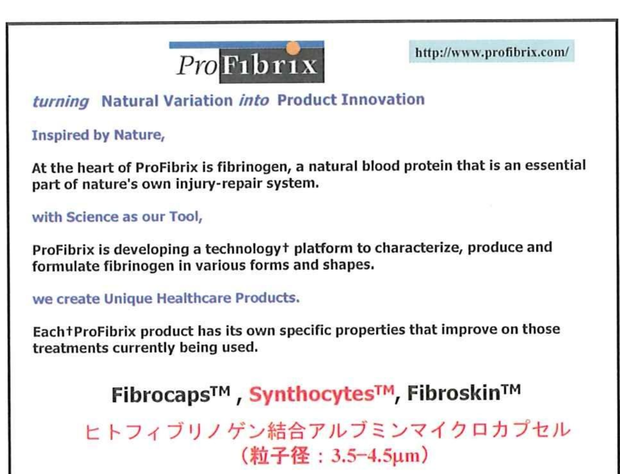
Thromboerythrocytes

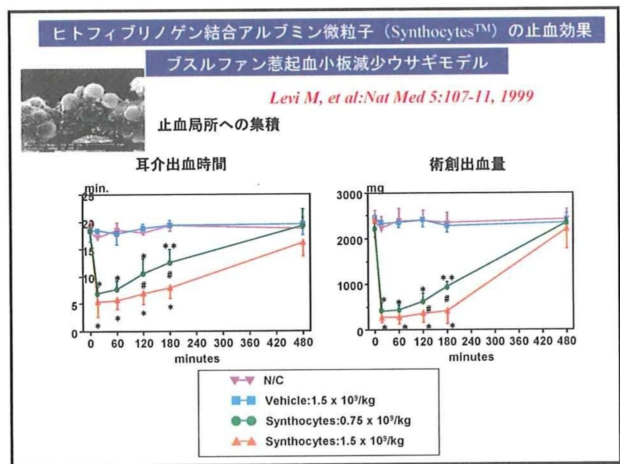
In Vitro Studies of a Potential Autologous, Semi-artificial Alternative to Platelet Transfusions

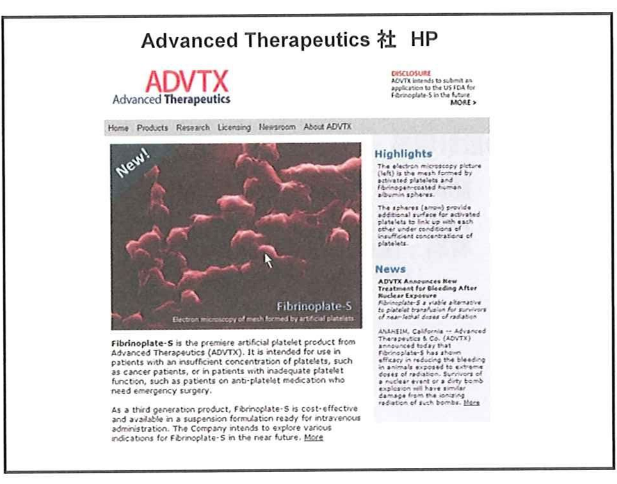
Barry S. Coller, Karen T. Springer, Juerg H. Beer, Narla Mohandas, Lesley E. Scudder, Karin J. Norton, and Sharon M. West
Division of Hematology, State University of New York at Stony Brook, Stony Brook, New York 11794; Laboratory for Thrombosis Research, University Hospital of Bern, CH-3010 Bern, Switzerland; and Division of Cell and Molecular Biology, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720











Fibrinoplate™

ヒトフィブリノゲン結合アルブミンマイクロスフェア

（粒子径：1.1-1.3μm）

臨床試験結果（フェーズII/III）

4th Asian Pacific Cong on Thromb & Haemost, 2006

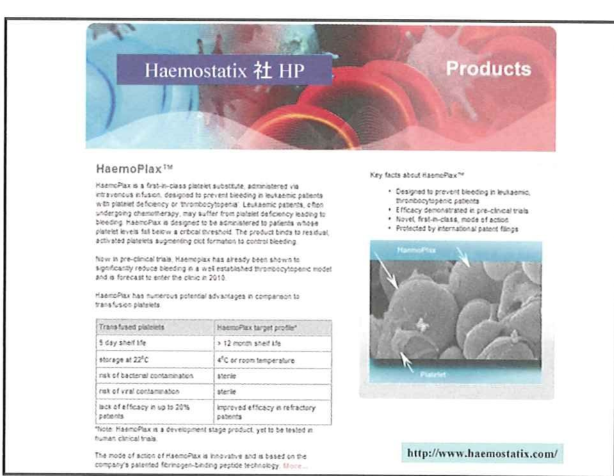
対象：血小板減少症（ITP、白血病、MDS、再生不良性貧血）
　　血小板数：＜3万／μl

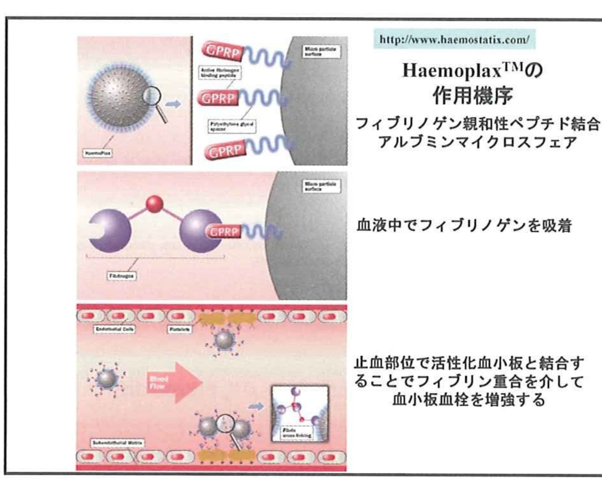
方法：double blind（試験物：7.0 mg/kg vs Fg非結合Alb粒子）

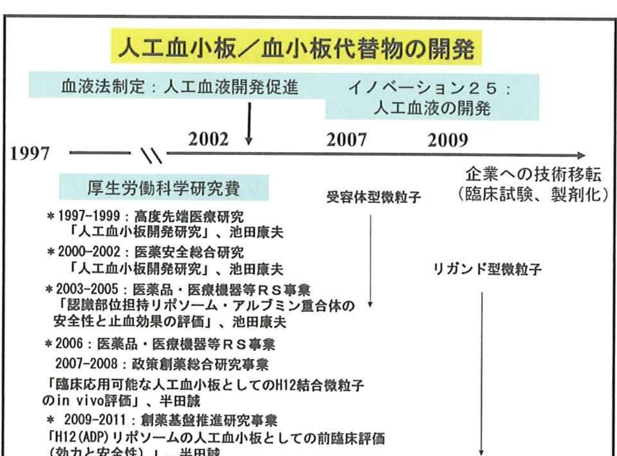
結果：N=102,223

出血時間の短縮効果（秒）

	0 hr vs 25 hr	0 hr vs 25hr
試験群	670.3 + 564.5	459.2 + 577.0
対照群	-55.5 + 199.1	-8.8 + 372.9
	p=0.000	p=0.042





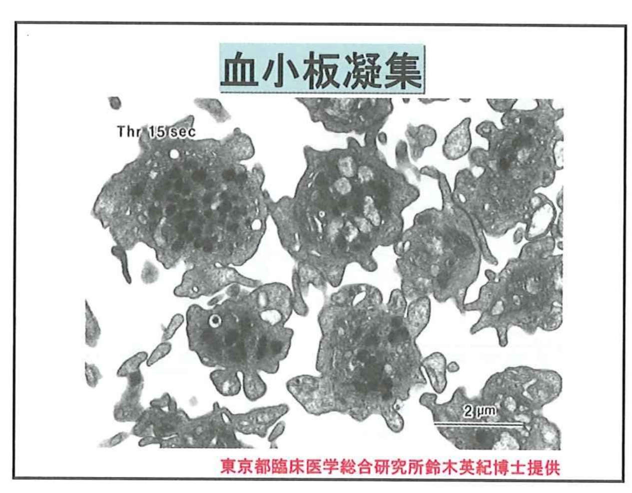


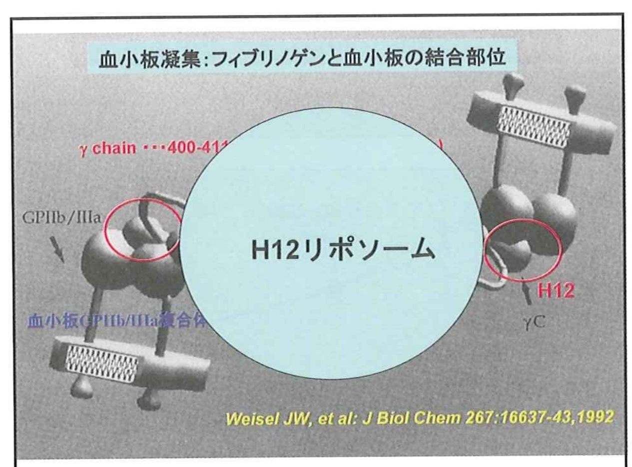
人工血小板

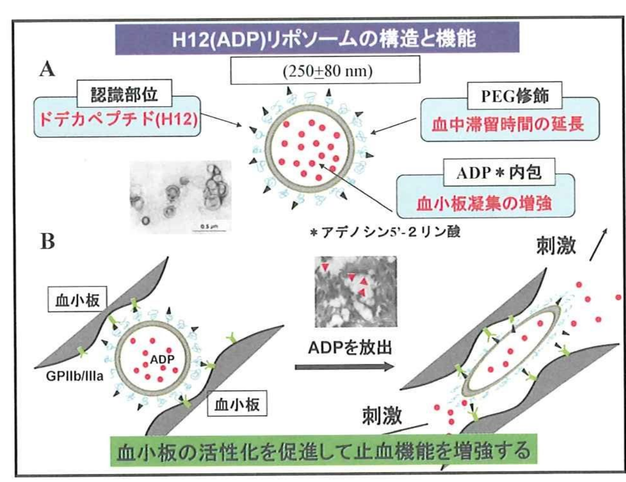
我々の設計戦略：

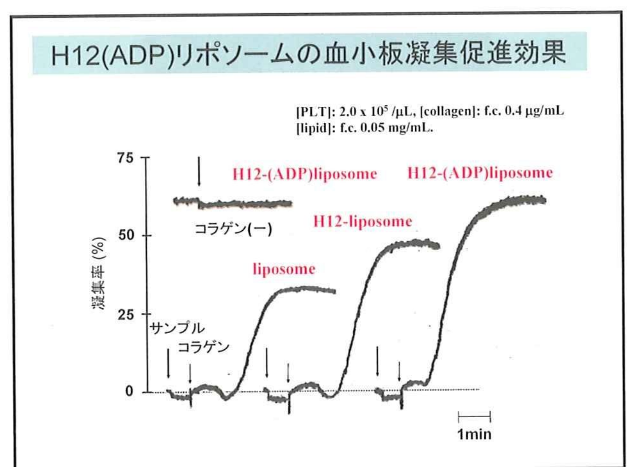
＃１　認識分子：血小板接着受容体とリガンド
＃２　担体：アルブミン重合体、リポソーム
＃３　材料：遺伝子組換体、合成物質
　　　（ヒト由来産物は使用せず）

完全な人工産物





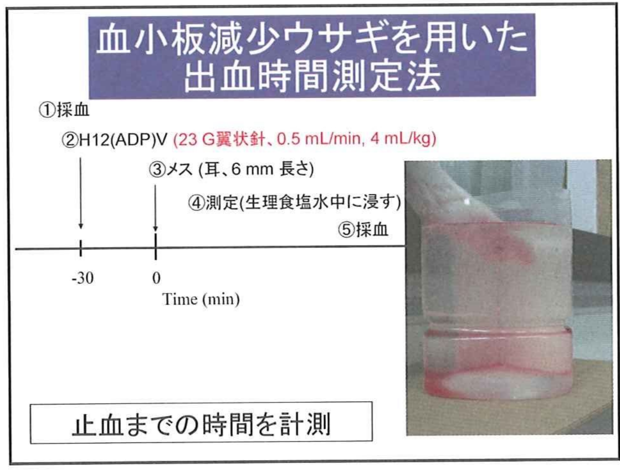


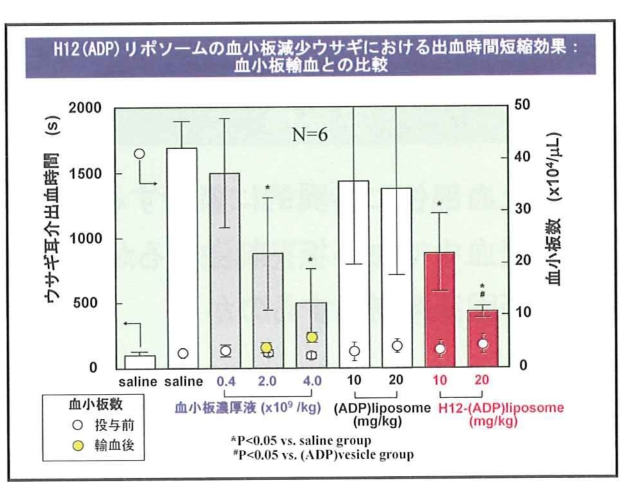


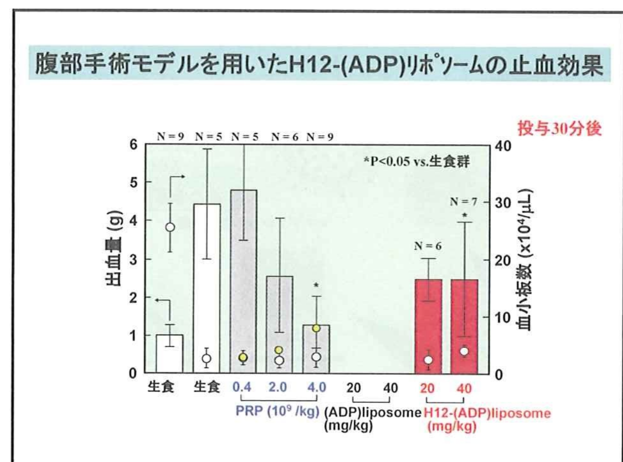
H12- (ADP)リポソームの止血機能：血小板減少ウサギでの検討

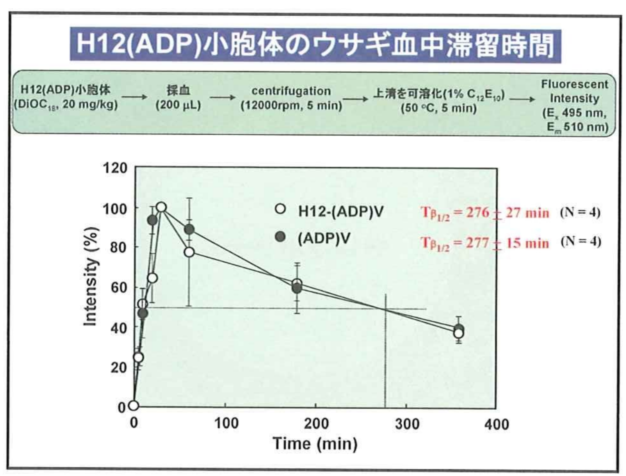
(1) 耳介出血時間の短縮

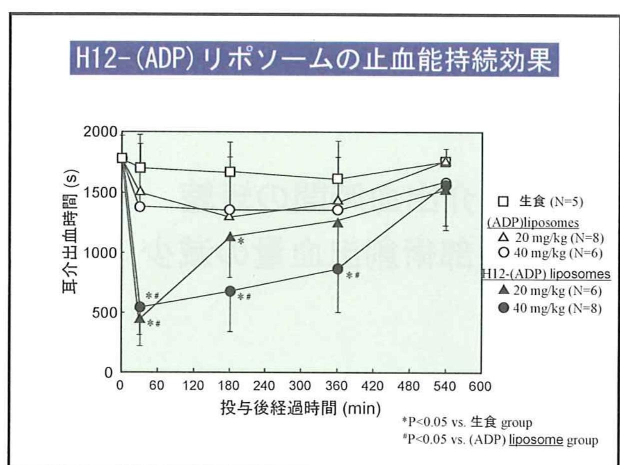
(2) 腹部術創出血量の減少

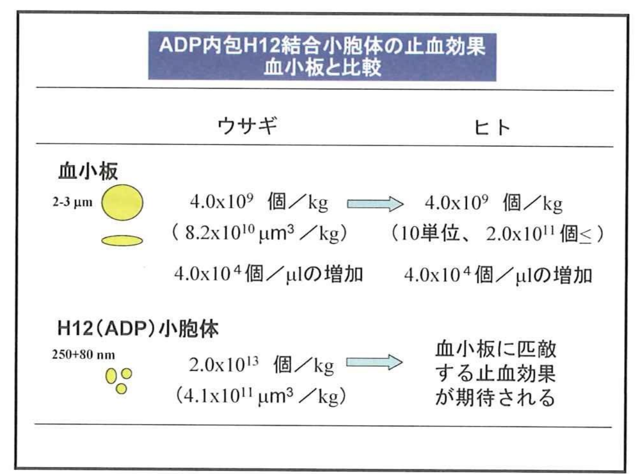










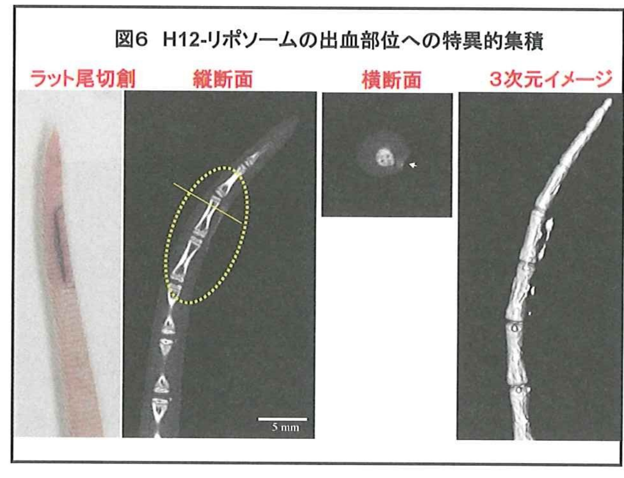


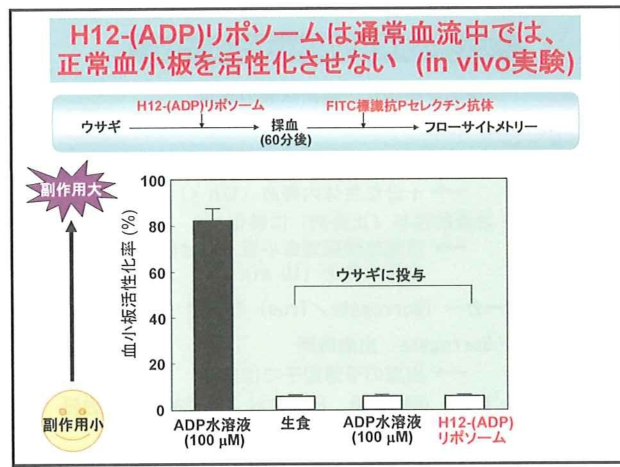
H12- (ADP)リポソームの安全性

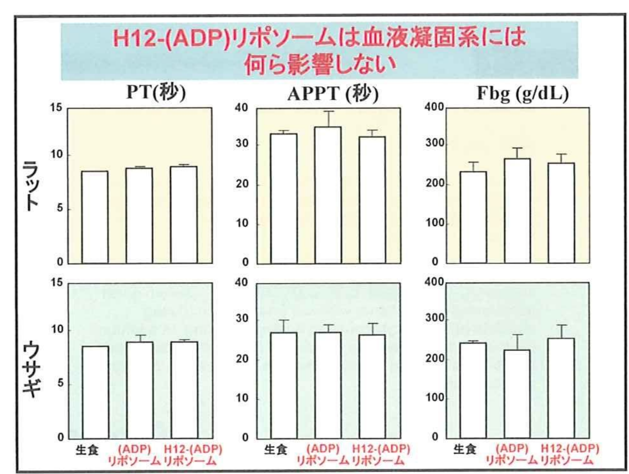
(1) 止血部位に特異的に集積するか？

(2) 流血中で血小板を刺激するか？

(3) 凝固系を刺激するのか？







我々の現在までの研究成果

Biomacromolecules 2:1192-1197 (2001), *Biochem Biophys Res Commun* 296:765-770 (2002), *ibid* 306:256-260 (2003), *ibid* 312:773-779 (2003), *Transfusion* 45 : 1221-1228 (2005), *Transfusion* 47 : 1254-1262 (2007), *Transfusion Med 18:158-166 (2008), J Thromb Haemost 7:470-477(2009)*

●血小板輸血に匹敵する止血効果（血小板減少動物）を示す人工微粒子（人工血小板：H12-(ADP)リポソーム）が創製された。

●人工血小板は十分な血中滞留性を保持し、生体内での血小板の活性化や血液凝固系への影響は認められなかった。

人工血小板代替物の過去・現在・未来

- □ 止血機構と血小板
- □ 人工血小板代替物の必要性
- □ 人工血小板代替物の条件
- □ 過去／現在の開発状況
- ■ 将来の展望

血小板代替物開発の変遷

1950 — 1995 — 2000 — 2005 — 2010

血小板由来産物 (platelet products)

- 凍結乾燥固定血小板（Stasix™） preclinical
- Infusible Platelet Membrane:IPM（Cyplex™） I — II → ×

人工血小板 (platelet substitutes)

- ヒトFg結合Albマイクロカプセル（Synthocytes™） I/II → ×
- ヒトFg結合Albマイクロスフェア（Fibrinoplate-S™） II/III
- Fg親和性ペプチド結合Albマイクロスフェア（Haemoplax™） preclinical
- H12ペプチド結合人工微粒子（日本） preclinical

ＩＰＭ（Cyplex™）の臨床効果

■ Phase I trial: Goodnough LT, et al: Blood (ab), 1995

健常ボランテア（２２人）への投与で安全性（急性毒性と免疫原性）確認

血小板減少患者（血小板数５万／μｌ未満で出血症状ナシ）への投与で有効性確認

出血時間短縮：６例／８例（７５%）

■ Phase II trial: Scigliano E, et al: Blood (ab), 1997

血小板減少患者（血小板数５万／μｌ未満で出血症状あり）への投与で有効性確認

症状の改善や止血：２７例／４０例（６５%）

人工血小板代替物開発推進のポイント

適応を明確化／臨床試験の想定

1）予防／治療

- 予防的投与（出血予防薬）にも対応
  → 十分な生体内寿命（12h ≦）
- 治療的投与（止血剤）に特化
  → 凍結乾燥固定血小板（Stasix™）：生体内寿命（10 min ≧）

2）マーカー（Surrogate／True）の設定

- Surrogate：出血時間
  → 出血の予想因子ではない
- True：便Hb定量、出血症状（他覚的）、出血量

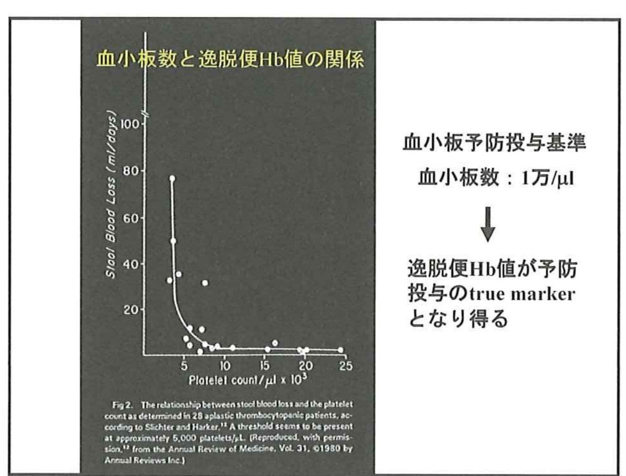
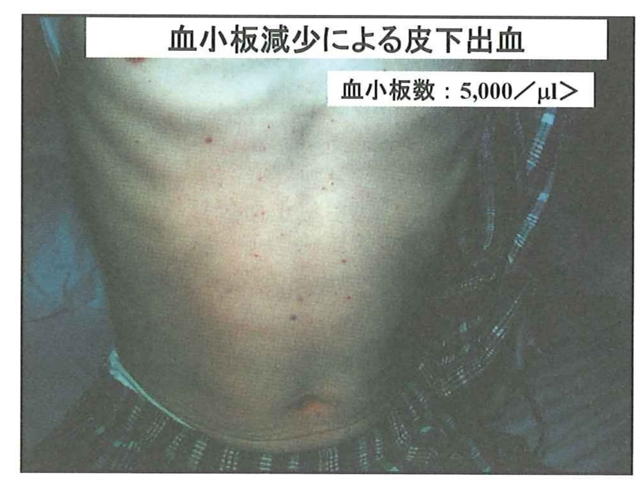
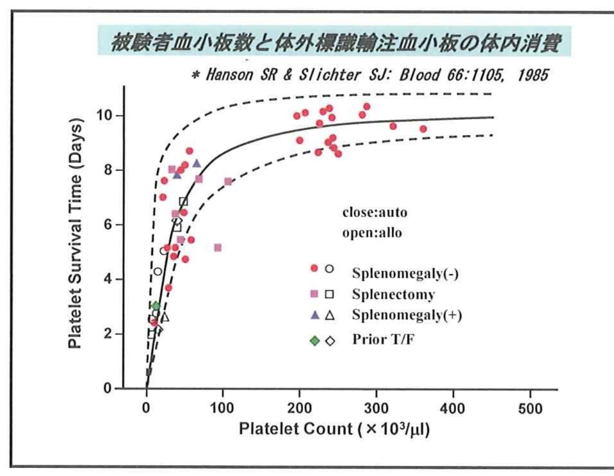


Fig 2. The relationship between stool blood loss and the platelet count as determined in 28 aplastic thrombocytopenic patients, according to Slichter and Harker.[13] A threshold seems to be present at approximately 5,000 platelets/μL. (Reproduced, with permission,[13] from the Annual Review of Medicine, Vol. 31, ©1980 by Annual Reviews Inc.)





血小板の体内動態

1）定常状態*

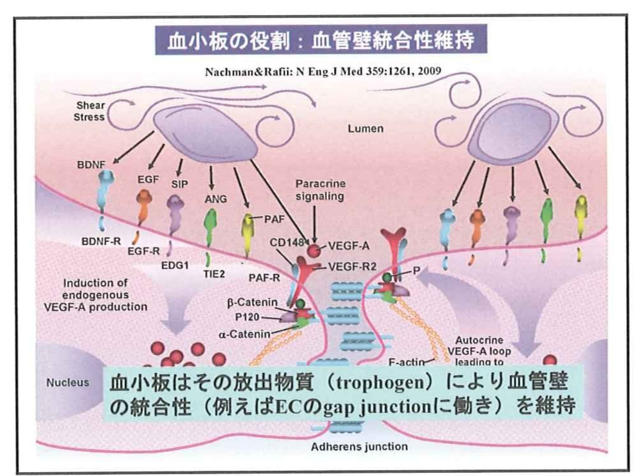
最大寿命：10.5日＝10%／日
統合性維持：0.71万*／μｌ／日
細胞回転：18%（ 4.12万／μｌ／日）
→ 血小板輸血：10単位／毎日

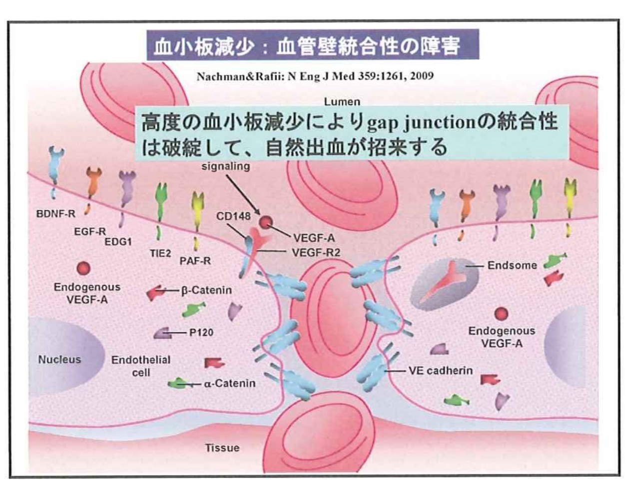
2）消費増大

出血、発熱、感染症、ＤＩＣ
（血小板輸血不応因子）
→ 血小板輸血：量／回数を追加

＊ Hanson SR & Slichter SJ: Blood 66:1105, 1985

*脾臓への停留量：34%を除外した場合は0.47万





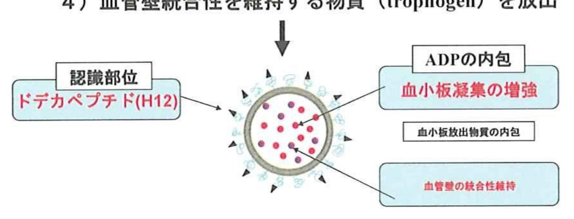
血小板予防投与を代替できる人工血小板の条件

1）十分な生体内寿命を持つ

2）活性化血小板と特異的に結合

3）十分な止血効果（止血血栓形成増強）を持つ

4）血管壁統合性を維持する物質（trophogen）を放出



人工血小板代替物：まとめ

その必要性

危機管理面ばかりでなく技術革新（ナノテク、DDSなど）の面からも医療に恩恵をもたらす。

その有望性

リガンド（フィブリノゲンとその関連ペプチド）結合アルブミン微粒子の検討が欧米で企業化されている。

将来展望

近い将来の製剤化を目指し、我が国独自の細胞型H12(ADP)小胞体の開発を推進してゆく。

平成18－20年度厚生労働科学研究費補助金

（政策創薬総合研究事業）

臨床応用可能な人工血小板としてのH12結合微粒子のin vivo評価

（主任研究者）　半田　誠　（慶應義塾大学医学部）

（分担研究者）　池田 康夫　（早稲田大学先進理工学）

武岡 真司　（早稲田大学先進理工学）

梶村 真弓　（慶應義塾大学医学部）

後藤 信哉　（東海大学医学部）

鈴木 英紀　（東京都臨床医学総合研究所）

村田　満　（慶應義塾大学医学部）

（研究協力者）　岡村 陽介　（早稲田大学先進理工学）

勝野 峻介　（早稲田大学先進理工学）