

those for commercial HSA are $54.31 \pm 8.23 \mu\text{L/h}$, $83.12 \pm 5.43 \mu\text{L/h}$ and $63.65 \pm 3.56 \mu\text{L/h}$, respectively. The slightly lower half-life and the slightly higher organ uptake clearances found for Alb A are most probably due to differences in isolation and/or defatting procedures.

The effects of the mutations on plasma half-lives and organ uptake clearances, calculated according to Eq.(3), are given in Table 2. As seen, all the proAlb variants and the truncated variants have diminished plasma half-lives, i.e., negative percent changes; this is most evident for Alb Venezia. By contrast, the effect of glycosylation varied: Almost no effect in the case of Alb Malmö-95 and Casebrook but a significant prolongation of the half-life for Alb Redhill. The increase of the half-life for Alb Redhill can partly be explained by the presence of -1 Arg (Table 2). In all 9 cases, the molecular changes resulted in a modified liver, kidney and spleen uptake clearance (Table 2). The most pronounced effects were found for the truncated variants Alb Bazzano and Venezia; the uptakes by the three organs were increased very much. At the other extreme, the uptakes of Alb Redhill were in all cases significantly decreased. For all three variants, the changes in organ uptakes can explain, at least partly, the changes in plasma half-lives. For the remaining six albumins, the effects on organ uptakes are more complex, i.e., both increases and decreases in uptake were found for the same variant. The most pronounced changes, i.e., more than 100%, were observed for liver and kidney uptakes of Alb Bazzano and Venezia.

3.3. Relationships between structure, stability and pharmacokinetic properties of HSA variants

The effects of genetic variation on the albumin structure were studied by far-UV intrinsic CD. From Fig. 3 it is apparent that the variants have spectra which differ to varying extents from that of their corresponding Alb A. In most cases, the structural modification results in a less negative spectrum. However, in two cases (Arg-Alb and Alb Venezia) the variant has a more negative spectrum. The most pronounced spectral changes were found for two variants having a modified chain length (proAlb Tradate and Alb Bazzano). We used the ellipticities at 222 nm to calculate the α -helical content of all the albumins. The impact of genetic variation on that parameter is given in a quantitative way in Table 2. Because the albumins had both modified pharmacokinetic properties (section 3.2.) and modified α -helical contents we investigated, whether there is a direct correlation between these parameters. That was found not to be the case. When plotting the changes in half-lives as a function of changes in α -helical content a poor correlation was found; a straight line had a P -value of 0.48 (not shown). Likewise, no correlations were found between the changes in organ uptake clearances and changes in α -helical content; the P -values were in the range 0.16–0.52 (not shown).

Previously, we have studied the effects of genetic variation on the thermal stability of HSA [15]. The stability was quantified by determining van't Hoff enthalpies (ΔH_f -values), and the results of that study are included in Table 2. We also examined whether a

correlation exists between changes in the ΔH_f -values and the changes in pharmacokinetic properties. However, no such correlation was found. When plotting the changes in half-lives versus the changes in ΔH_f -values, the P -value was only 0.68 (not shown). In addition, no correlations were found between the changes in organ uptakes and changes in ΔH_f -values; the P -values were in the range 0.38–0.83 (not shown).

4. Discussion

Clinically, it would be useful, if protein engineering could result in the production of HSA preparations with a prolonged half-life in the circulation. In addition, because of its half-life of 19 days in humans, its ease of synthesis and its known structure albumin is an attractive candidate for use in recombinant fusion proteins and as a carrier in drug delivery systems. However, also in the two latter types of examples it would be advantageous to be able to modify the plasma half-life of the protein product. For finding such isoforms, in the present study, we investigated the half-life and organ uptakes of a series of genetic HSA variants with relatively large molecular changes, i.e., modified chain-length or glycosylated. The most clear results were obtained for two truncated albumins (Alb Venezia and Bazzano) and a glycosylated variant (Alb Redhill). For the other six variants less clear and individual results were obtained.

All the albumins with chain-lengths deviating by three or more residues from Alb A have diminished half-lives in the circulation (Table 2). This is most evident for Alb Bazzano and Venezia the half-lives of which were reduced by 6–9%. These results must be due to mutation induced changes in protein charge and/or conformation. In this connection it is relevant to note that Alb Bazzano has lost its C-terminal disulfide bridge because of the 567 Cys \rightarrow Ala substitution (see Section 3.1.). Our previous study [14], making use of single-residue mutations, revealed that the half-life of Alb Hawkes Bay is shortened by ca. 30%. This finding is due to the mutation 177 Cys \rightarrow Phe [33], which results in the loss of the disulfide bond between 168 Cys and 177 Cys. Thus, the lifespan of HSA is dependent on the existence of its 17 stabilizing disulfide bridges [1–4].

The effect of glycosylation depends on the position of attachment. Glycosylation of 63 Asn in domain I (Alb Malmö-95) or 494 Asn in domain III (Alb Casebrook) has no significant effect on variant half-life. By contrast, glycosylation of 318 Asn in domain II (Alb Redhill) results in a significant increase in half-life; however, part of the increment is caused by the presence of -1 Arg. Sheffield et al. [9] also observed position-dependent effects of *N*-glycosylation when studying the half-life of mutated rabbit serum albumin in rabbits. These authors reported no effect of glycosylation of 12 Asn in the 14 Val \rightarrow Thr variant. By contrast, a similar modification of 494 Asn in the 494 Asp \rightarrow Asn isoform resulted in a reduction of the mean terminal catabolic half-life from 4.32 days to 2.87 days. The different results obtained in our and their study for the latter variant is probably due to species differences between protein and/or test animal.

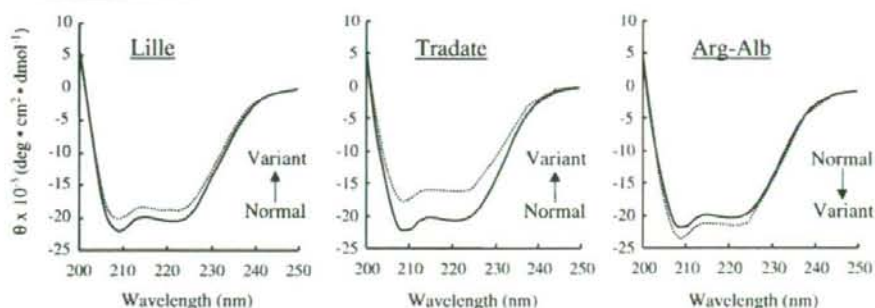
Table 2
Percent changes of half-life and organ uptake clearances of HSA variants in mice

Variant name (mutation)	Half-life (%)	Liver clearance (%)	Kidney clearance (%)	Spleen clearance (%)	α -helical content (%)	ΔH_f^a (%)
proAlb Lille (-2Arg \rightarrow His)	-4.99	31.58	-24.63	-8.48	-9.60	-6.08
proAlb Tradate (-2Arg \rightarrow Cys)	-1.19	1.82	93.74	-14.84	-24.15	N.D. ^b
Arg-Alb (Alb A having -1 Arg)	3.39	-77.58	-16.25	15.75	5.64	3.80
Alb Bazzano (567-582 substituted, 583-585 deleted)	-5.73	358.94	111.49	46.10	-20.09	-4.49
Alb Venezia (572-578 substituted, 579-585 deleted)	-8.94	225.74	116.50	30.32	5.41	34.50
Alb Catania (580-582 substituted, 583-585 deleted)	-1.07	-74.92	71.83	-12.75	-1.09	-19.71
Alb Malmö-95 (63 Asp \rightarrow Asn, glycosylated at 63 Asn)	-1.10	82.91	2.07	-35.65	-0.31	-30.75
Alb Redhill (-1 Arg retained, 320 Ala \rightarrow Thr, glycosylated at 318 Asn)	6.16	-72.52	-50.24	-45.63	-3.26	-4.48
Alb Casebrook (494 Asp \rightarrow Asn, glycosylated at 494 Asn)	0.84	65.49	-5.17	-1.36	-7.06	17.54

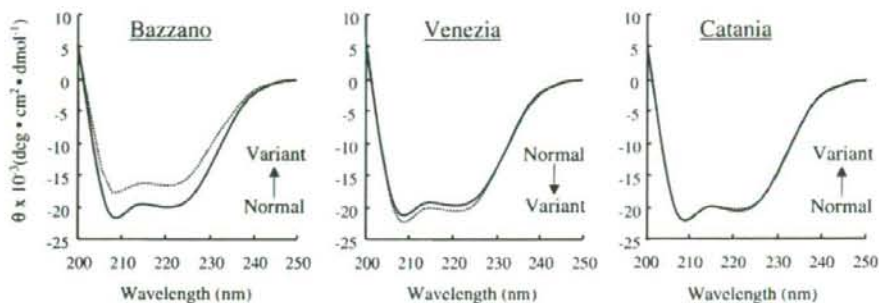
^a The values for ΔH_f are taken from Kragh-Hansen et al. [15].

^b ND, Not determined.

(A) ProAlb variants



(B) Truncated HSA variants



(C) Glycosylated HSA variants

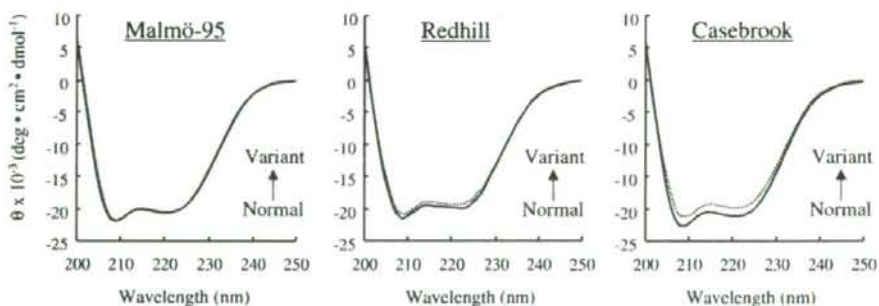


Fig. 3. Far-UV CD spectra of proAlb variants (A), truncated HSA variants (B) and glycosylated HSA variants (C) and their corresponding Alb A. The dotted and full curves represent variant and normal albumin, respectively. The curves are averages for three experiments.

In addition to change in chain-length and glycosylation we investigated whether the modified plasma half-lives could be correlated to other molecular parameters. Therefore, we estimated the effect of genetic variation on the α -helical content of albumin and related any changes to the changes in plasma half-life and organ uptakes. However, no clear correlation could be found between the changes in α -helical content and the different pharmacokinetic parameters (not shown). The changes in half-life and organ uptakes were also compared to mutation-induced changes in heat stability; quantified by using ΔH_v [15]. However, also in this case no correlations were found (not shown).

For being able to explain, at least in part, the modified plasma half-lives of the genetic variants we investigated their liver, kidney and spleen uptake clearances (Table 1). The diminished plasma half-lives of Alb Bazzano and Venezia are in full accordance with pronounced increases in liver, kidney and spleen uptake clearances. Likewise, the

increased half-life of Alb Redhill can fully, or partly, be caused by diminished uptake by the three organs. Although most of the remaining variants have an increased liver clearance and a reduced spleen uptake individual results were obtained for these six alloalbumins. However, for all variants also other metabolic factors could be modified, see below.

Uptake of HSA by liver, kidney and spleen is mainly due to the presence of cell membrane receptors which recognize the protein and then internalize it by endocytosis. Some of these receptors interact with native protein, whereas other receptors interact with modified protein, and both types of interactions could be affected by genetic variation of albumin. Thus, binding of HSA to the membrane-bound receptor gp60 initiating a transcellular pathway for albumin across the endothelial cell wall to the underlying interstitium [34] could be affected. Sometimes, a glycosylated albumin variant loses one or both of the sialic acid residues on the antennae. Since the remaining of

the glycans has galactose and mannose units, these forms could interact with increased affinity with the galactosyl receptor-mediated (asialoglycoprotein receptor-mediated) endocytotic pathway of the hepatocytes and the mannose receptor-mediated endocytotic pathway of non-parenchymal cells in the liver. The liver also possesses receptors for rapid uptake of oxidized albumin and albumin with advanced glycation end products. Whether the present molecular changes can initiate endocytosis by scavenger receptors such as gp18, gp30, stabilin-1 or stabilin-2 is at present only speculative. However, liver uptake by adsorptive endocytosis could be influenced by the molecular changes, because this type of uptake is dependent on the net charge of the protein.

Normally, glomerular filtration of HSA in the kidneys is followed by its return into the venous circulation without degradation (the albumin retrieval pathway). However, part of the protein reabsorbed by the proximal tubule cells, most probably via the endocytic receptors megalin and cubulin, is degraded in the lysosomes. Whether genetic modification of HSA affects glomerular filtration and/or reabsorption of the protein remains to be clarified. However, since the size and charge of albumin influence glomerular filtration, especially the glycosylated variants could have an altered handling by the glomeruli. It should be noted that no radioactivity was detected in the urine during the present experimental period. An alternative, or supplementary, explanation for the modified uptake of the albumin isoforms by the kidney could be the presence of tubular RAGE which is known to cause the internalization of proteins with advanced glycation end products. Irrespective of the detailed mechanism, the net effect of deleting three or more C-terminal amino acids is an increased kidney uptake clearance (Tables 1 and 2). The effect of glycosylation depends on the position of attachment, because glycosylation of domain II (Alb Redhill) decreases uptake, whereas glycosylation of domain I (Alb Malmö-95) and domain III (Alb Casebrook) has no significant effect on kidney uptake.

Glycosylation diminishes uptake by the spleen (Tables 1 and 2). This is most evident for Alb Redhill, especially when taking into account that the presence of -1 Arg increases uptake. The presence of a modified propeptide also decreases clearance uptake. By contrast, the truncated variants with the most pronounced molecular changes (Alb Bazzano and Venezia) have significantly increased uptakes. Apparently, less is known about endocytosis-associated membrane receptors in the spleen than in the liver and kidneys. However, the organ most probably also has endothelial cells with the receptor gp60 [34]. In addition, it harbors scavenger receptors such as stabilin-1 and stabilin-2. To what extent albumin-receptor interactions are affected by the mutations remains to be elucidated.

More recently, another type of endocytosis of HSA has been identified in virtually all nucleated cells which results in reuse of the protein [35–37]. After pinocytosis, albumin binds intracellularly and in a pH-dependent manner to the receptor FcRn. Thereby the protein is diverted from the lysosomal degradation pathway and exocytosed back to the circulation in an intact form extending its plasma half-life. Anderson et al. [35] have proposed that the intracellular binding of HSA to FcRn is due to a hydrophobic interaction, whereas Chaudhury et al. [36] suggested interaction(s) between histidine residue(s) in the receptor and histidine residues in domain III of albumin. By contrast, Andersen et al. [37] suggested that FcRn interacts with negatively charged and surface exposed residues on domain III of HSA. However, the authors cited agree that domain III of albumin seems to contain all of the FcRn binding activity, why especially genetic variants with domain III changes could have modified plasma half-lives due to a modified HSA–FcRn recycling process.

The metabolism of HSA can be affected by enzymes such as aminopeptidase(s) and carboxypeptidase(s) in the circulation. That has been observed in, for example, a patient with a severe traumatic injury, who had an increased activity of carboxypeptidase A resulting

in hydrolysis of the C-terminal leucine and a fast elimination of albumin from the blood; the half-life was changed from ca. 19 days to less than 80 h [38]. In addition, modified albumin can be a substrate for endopeptidase(s) in the blood. Thus, oxidized bovine serum albumin, but not the native protein, can be cleaved by oxidized protein hydrolase [39]. Because the hydrolase is found in the blood, also this enzyme could hydrolyse some of the genetic variants in the mouse circulation and thereby render them more exposed to organ uptake.

In conclusion, the pharmacokinetics of HSA can be modified by changes in chain-length and by glycosylation. Three of the alloalbumins with a modified chain-length had a significantly shorter half-life in the circulation, whereas a glycosylated protein had an increased half-life (Table 1). These findings are useful when trying to construct an isoform with a modified stability in plasma. If an isoform with a shorter half-life is wanted, it is probably preferable to choose one with a C-terminally shortened chain-length rather than one with a N-terminally elongated chain-length, because the presence of a propeptide is known to block the high-affinity binding site for metal ions such as Cu^{2+} and Ni^{2+} [40]. It is a disadvantage to block this binding site, because albumin binding of Cu^{2+} is an element in our anti-oxidative defense. Liver, kidney and spleen clearances were determined, and eight of the nine genetic variants had one or more modified organ uptakes. The uptakes measured are net effects of organ uptake leading to destruction in the lysosomes or to recycling via the FcRn receptor. The uptakes themselves and the two intracellular pathways could be affected differently by the protein modifications. The results revealed that if an increased uptake in the three organs is wanted for, for example, drug delivery systems, then truncated variants like Alb Bazzano and Venezia are good candidates. If the opposite is wanted, namely decreased uptakes in the organs, then the glycosylated Alb Redhill could be useful. Finally, we investigated whether blood half-lives or organ uptakes are directly related to mutation-induced changes in the proteins α -helical content or to changes in ΔH_m , representing thermal stability. However, that was found not to be the case.

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References

- [1] U. Kragh-Hansen, Molecular aspects of ligand binding to serum albumin, *Pharmacol. Rev.* 33 (1981) 17–53.
- [2] T. Peters Jr., *All About Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego, CA, 1996.
- [3] D.C. Carter, J.X. Ho, Structure of serum albumin, *Adv. Protein Chem.* 45 (1994) 153–203.
- [4] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 Å resolution, *Protein Eng.* 12 (1999) 439–446.
- [5] M.L. Ferrer, R. Duchowicz, B. Carrasco, J.G. de la Torre, A.U. Acuna, The conformation of serum albumin in solution: a combined phosphorescence depolarization-hydrodynamic modeling study, *Biophys. J.* 80 (2001) 2422–2430.
- [6] K. Kobayashi, Summary of recombinant human serum albumin development, *Biologicals* 34 (2006) 55–59.
- [7] S. Matsushita, V.T.C. Chuang, M. Kanazawa, S. Tanase, K. Kawai, T. Maruyama, A. Suenaga, M. Otogiri, Recombinant human serum albumin dimer has high blood circulation activity and low vascular permeability in comparison with native human serum albumin, *Pharm. Res.* 23 (2006) 882–891.
- [8] T. Komatsu, Y. Oguro, Y. Teramura, S. Takeoka, J. Okai, M. Anraku, M. Otogiri, E. Tsuchida, Physicochemical characterization of cross-linked human serum albumin dimer and its synthetic heme hybrid as an oxygen carrier, *Biochim. Biophys. Acta* 1675 (2004) 21–31.
- [9] W.P. Sheffield, J.A. Marques, V. Bhakta, I.J. Smith, Modulation of clearance of recombinant serum albumin by either glycosylation or truncation, *Thromb. Res.* 99 (2000) 613–621.
- [10] G.M. Subramanian, P.A. Moore, B.B. Gowen, A.L. Olsen, D.L. Barnard, J. Paragas, R.J. Hogan, R.W. Sidwell, Potent in vitro activity of the albumin fusion type 1 interferons (albumin-interferon- α and albumin-interferon- β) against RNA viral agents of bioterrorism and the severe acute respiratory syndrome (SARS) virus, *Chemotherapy* 54 (2008) 176–180.

- [11] J. Chen, G. Bai, Y. Cao, Z. Gao, Q. Zhang, Y. Zhu, W. Yang, One-step purification of a fusion protein of glucagon-like peptide-1 and human serum albumin expressed in *Pichia pastoris* by an immunomagnetic separation technique, *Biosci. Biotechnol. Biochem.* 71 (2007) 2655–2662.
- [12] R.J. Melder, B.L. Osborn, T. Riccobene, P. Kanakaraj, P. Wei, G. Chen, D. Stolow, W.G. Halpern, T.-S. Migone, Q. Wang, K.J. Grzegorzewski, C. Gallant, Pharmacokinetics and in vitro and in vivo anti-tumor response of an interleukin-2-human serum albumin fusion protein in mice, *Cancer Immunol. Immunother.* 54 (2005) 535–547.
- [13] L. Minchiotti, M. Galliano, U. Kragh-Hansen, T. Peters Jr., Mutations and polymorphisms of the gene of the major human blood protein, serum albumin, *Hum. Mutat.* 29 (2008) 1007–1016.
- [14] Y. Iwao, M. Hiraiki, U. Kragh-Hansen, K. Mera, T. Noguchi, M. Anraku, K. Kawai, T. Maruyama, M. Otagiri, Changes of net charge and α -helical content affect the pharmacokinetic properties of human serum albumin, *Biochim. Biophys. Acta* 1774 (2007) 1582–1590.
- [15] U. Kragh-Hansen, S. Saito, K. Nishi, M. Anraku, M. Otagiri, Effect of genetic variation on the thermal stability of human serum albumin, *Biochim. Biophys. Acta* 1747 (2005) 81–88.
- [16] U. Kragh-Hansen, A micromethod for delipidation of aqueous proteins, *Anal. Biochem.* 210 (1993) 318–327.
- [17] R.F. Chen, Removal of fatty acids from serum albumin by charcoal treatment, *J. Biol. Chem.* 242 (1967) 173–181.
- [18] D.J. Hnatowich, W.W. Layne, R.L. Childs, The preparation and labelling of DTPA-coupled albumin, *Int. J. Appl. Radiat. Isot.* 33 (1982) 327–332.
- [19] F. Staud, M. Nishikawa, K. Morimoto, Y. Takakura, M. Hashida, Disposition of radioactivity after injection of liver-targeted proteins labeled with ^{111}In or ^{125}I . Effect of labeling on distribution and excretion of radioactivity in rats, *J. Pharm. Sci.* 88 (1999) 577–585.
- [20] J.R. Duncan, M.J. Welch, Intracellular metabolism of indium-111-DTPA-labeled receptor targeted proteins, *J. Nucl. Med.* 34 (1993) 1728–1738.
- [21] K. Yamaoka, Y. Tanigawara, T. Nakagawa, T. Uno, A pharmacokinetic analysis program (multi) for microcomputer, *J. Pharmacobiodyn.* 4 (1981) 879–885.
- [22] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [23] Y.-H. Chen, J.T. Yang, H.M. Martinez, Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion, *Biochemistry* 11 (1972) 4120–4131.
- [24] Y. Abdo, J. Rousseaux, M. Dautrevaux, Proalbumin Lille, a new variant of human serum albumin, *FEBS Lett.* 131 (1981) 286–288.
- [25] S.O. Brennan, K. Arai, J. Madison, C.-B. Laurell, M. Galliano, S. Watkins, R. Peach, T. Myles, P. George, F.W. Putnam, Hypermutability of CpG dinucleotides in the propeptide-encoding sequence of the human albumin gene, *Proc. Natl. Acad. Sci. U. S. A.* 87 (1990) 3909–3913.
- [26] S. Watkins, J. Madison, E. Davis, Y. Sakamoto, M. Galliano, L. Minchiotti, F.W. Putnam, A donor splice mutation and a single-base deletion produce two carboxyl-terminal variants of human serum albumin, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 5959–5963.
- [27] J. Madison, M. Galliano, S. Watkins, L. Minchiotti, F. Porta, A. Rossi, F.W. Putnam, Genetic variants of human serum albumin in Italy: point mutants and a carboxyl-terminal variant, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 6476–6480.
- [28] Y. Sakamoto, K. Kitamura, J. Madison, S. Watkins, C.-B. Laurell, M. Nomura, T. Higashiyama, F.W. Putnam, Structural study of the glycosylated and unglycosylated forms of a genetic variant of human serum albumin (63 Asp→Asn), *Biochim. Biophys. Acta* 1252 (1995) 209–216.
- [29] S.O. Brennan, T. Myles, R.J. Peach, D. Donaldson, P.M. George, Albumin Redhill (-1 Arg, 320 Ala→Thr): a glycoprotein variant of human serum albumin whose precursor has an aberrant signal peptidase cleavage site, *Proc. Natl. Acad. Sci. U. S. A.* 87 (1990) 26–30.
- [30] U. Kragh-Hansen, D. Donaldson, P.H. Jensen, The glycan structure of albumin Redhill, a glycosylated variant of human serum albumin, *Biochim. Biophys. Acta* 1550 (2001) 20–26.
- [31] R.J. Peach, S.O. Brennan, Structural characterization of a glycoprotein variant of human serum albumin: albumin Casebrook (494 Asp→Asn), *Biochim. Biophys. Acta* 1097 (1991) 49–54.
- [32] P.A. Haynes, M. Batley, R.J. Peach, S.O. Brennan, J.W. Redmond, Characterization of oligosaccharides from a glycoprotein variant of human serum albumin (albumin Casebrook) using high-performance anion-exchange chromatography and nuclear magnetic resonance spectroscopy, *J. Chromatogr.* 581 (1992) 187–193.
- [33] S.O. Brennan, A.P. Fellowes, Albumin Hawkes Bay: a low level variant caused by loss of a sulphhydryl group at position 177, *Biochim. Biophys. Acta* 1182 (1993) 46–50.
- [34] T.A. John, S.M. Vogel, C. Tirupathi, A.B. Malik, R.D. Minshall, Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer, *Am. J. Physiol. Lung Cell Mol. Physiol.* 284 (2003) L187–L196.
- [35] C.L. Anderson, C. Chaudhury, J. Kim, C.L. Bronson, M.A. Wani, S. Mohanty, Perspective - FcRn transports albumin: relevance to immunology and medicine, *Trends Immunol.* 27 (2006) 343–348.
- [36] C. Chaudhury, C.L. Brooks, D.C. Carter, J.M. Robinson, C.L. Anderson, Albumin binding to FcRn: distinct from the FcRn-IgG interaction, *Biochemistry* 45 (2006) 4983–4990.
- [37] J.T. Andersen, J.D. Qian, I. Sandlie, The conserved histidine 166 residue of the human neonatal Fc receptor heavy chain is critical for the pH-dependent binding to albumin, *Eur. J. Immunol.* 36 (2006) 3044–3051.
- [38] D. Bar-Or, L.T. Rael, R. Bar-Or, D.S. Slone, M.L. Craun, The formation and rapid clearance of a truncated albumin species in a critically ill patient, *Clin. Chem. Acta* 365 (2006) 346–349.
- [39] T. Fujino, M. Kojima, M. Beppu, K. Kikugawa, H. Yasuda, K. Takahashi, Identification of the cleavage sites of oxidized protein that are susceptible to oxidized protein hydrolase (OPH) in the primary and tertiary structures of the protein, *J. Biochem.* 127 (2000) 1087–1093.
- [40] U. Kragh-Hansen, S.O. Brennan, L. Minchiotti, M. Galliano, Modified high-affinity binding of Ni^{2+} , Ca^{2+} and Zn^{2+} to natural mutants of human serum albumin and proalbumin, *Biochem. J.* 301 (1994) 217–223.



NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers

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ABSTRACT

Hemoglobin vesicles (HbVs) are artificial oxygen carriers encapsulating purified and concentrated Hb solution in phospholipid vesicles (liposomes). We examined in-vitro reaction profiles of a formulation of HbV with NO and CO in anaerobic and aerobic conditions using stopped-flow spectrophotometry and a NO electrode. Reaction rate constants of NO to deoxygenated and oxygenated HbV were considerably smaller than those of cell-free Hb because of the intracellular NO-diffusion barrier. The reaction of CO with deoxygenated HbV was slightly slower than that of cell-free Hb solely because of the co-encapsulated allosteric effector, pyridoxal 5'-phosphate. The NO depletion in an aerobic condition in the presence of empty vesicles was monitored using a NO electrode, showing that the hydrophobic bilayer membrane of HbV, which might have higher gas solubility, does not markedly facilitate the O₂ and NO reaction, and that the intracellular Hb is the major component of NO depletion. In conclusion, HbV shows retarded gas reactions, providing some useful information to explain the absence of vasoconstriction and hypertension when they are intravenously injected.

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1. Introduction

A physiologically important aspect of the structure of red blood cells (RBCs) is the management of the transport of endogenous gaseous messenger molecules (NO and CO) [1–3]. Conditions of hemolysis [4] and studies related to the development of hemoglobin-based oxygen carriers (HBOCs) [5–11] have shown that the entrapment of endothelium-derived NO induces vasoconstriction, hypertension, reduced blood flow, and vascular damage. Physiological doses of CO are a vasorelaxation factor, especially in the hepatic microcirculation [12] and its entrapment by cell-free hemoglobin (Hb) solutions induces constriction of sinusoidal capillaries [13]. These side effects due to the presence of molecular Hb in plasma suggest that the cellular structure of RBCs plays a role in insuring the bioavailability of NO and CO.

Several mechanisms have been proposed to explain NO binding retardation by Hb encapsulation in RBCs [14–16], namely: (i) an unstirred layer forming an extracellular diffusion barrier surrounding the RBC [2,14]; (ii) a protein-rich RBC cytoskeletal submembrane constituting a physical barrier against NO diffusion [17,18]; and (iii)

retardation of gas diffusion due to the viscosity of the Hb solution in RBCs [19].

Hemoglobin has been encapsulated using lipid bilayer membranes to form Hb vesicles (HbV), in order to produce a blood like HBOC where the oxygen carrying Hb is not dissolved in plasma [20,21]. In this configuration Hb is not vasoactive [9,10], an effect in part due to the retardation of NO binding by Hb encapsulation in phospholipid vesicles [22]. Furthermore, using results of stopped-flow spectrophotometry and computer simulation, we recently determined that the major component of retardation is the intracellular rather than the extracellular diffusion barrier. This conclusion is also supported by considering that: (i) the large binding rate constant of NO to a heme in an Hb molecule, (ii) the numerous hemes as sites of gas entrapment at a high intracellular Hb concentration [Hb]_{in}, (iii) the slowed gas diffusion in the intracellular viscous Hb solution; and (iv) the lipid bilayer membrane of HbV would not possess any barrier function to the NO diffusion into the particles [23].

On the other hand, the hydrophobic part of a cell membrane reportedly possesses higher gas solubility than the bulk aqueous phase. Both NO and O₂ dissolve to a greater extent in the hydrophobic part, which facilitates the reaction of NO with O₂ to produce NO₂ [24]. HbV mimics the structure of RBCs, however the particle diameter is much smaller than that of RBCs, and the surface to volume ratio is larger. Therefore for the same oxygen carrying capacity, or equivalent cell concentration by volume there is proportionally greater amount of membrane material for HbVs than for RBCs providing a greater

Abbreviations: HbV, hemoglobin vesicles; RBC, red blood cells; $k_{on}^{(NO)}$, apparent NO binding rate constant; $k_{on}^{(CO)}$, apparent CO binding rate constant; $k_{off}^{(NO)}$, apparent oxidation rate by NO; PLP, pyridoxal 5'-phosphate; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine

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hydrophobic domain. This structural difference is important because it is likely that there is NO depletion in the lipid bilayer membrane of HbVs.

In the present study we analyzed the NO and CO binding rates of HbV with the present formulation, along with the possibility of accelerated NO depletion in the hydrophobic domain of the HbV lipid bilayer membrane. We compared our findings to the corresponding binding rates of a purified human Hb solution suspended in phosphate buffered saline solution (PBS) mixed with pyridoxal 5'-phosphate (PLP), a solution of polymerized bovine Hb (poly_βHb) used for veterinary purposes (Oxyglobin; Biopure Corp. Cambridge, MA) [25], and vesicles containing saline solution.

2. Materials and methods

2.1. Hb vesicles (HbV), Hb solutions, empty vesicles (EV), and RBCs

HbVs were prepared as reported previously [26–29], with slight modifications. Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). Hb was stabilized by carbonylation (HbCO) and concentrated by ultrafiltration to 38 g/dL. PLP (Sigma, St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer=2.5. We use PLP instead of 2,3-diphosphoglyceric acid (2,3-DPG) or inositol hexaphosphate (IHP) because 2,3-DPG is very unstable and expensive, and the interaction of IHP and Hb is so strong that the oxygen dissociation curve of Hb is distorted and the Hill number becomes nearly 1 [30].

The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer was comprised of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-O-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG₅₀₀₀ (DSPE-PEG; NOF Corp., Tokyo, Japan) at a molar composition of 5/5/1/0.033. Particle diameter was regulated by extrusion. The encapsulated HbCO was converted to HbO₂ by exposing the liquid membrane of HbV to visible light under an O₂ atmosphere. The particle size distribution was measured using a light-scattering method (Submicron Particle Size Analyzer, model N4 PLUS; Beckman-Coulter, Inc., Fullerton, CA). An empty vesicle (EV) suspension was prepared using the same lipids through hydration with a saline solution. The lipid concentration (6.8 g/dL) and the particle diameter (ca. 250 nm) were almost identical to those of HbV.

A purified human Hb solution suspended in phosphate buffered saline solution (PBS) was prepared and mixed with PLP at molar ratios of PLP/Hb tetramer=0 and 2.5. We also used the polymerized bovine Hb solution (poly_βHb) (Oxyglobin; Biopure Corp. Cambridge, MA) [25]. This solution is a mixture of nonpolymerized tetrameric α Hb (37.2%) and polymerized α Hb with a wide molecular weight distribution [25]. The P₅₀ values and Hill numbers of HbV and Hb solutions were obtained from the oxygen equilibrium curve measured using a Hemox-Analyzer (TCS Medical Science, Philadelphia, PA) at 37 °C [23,31] (Fig. 1).

Blood was harvested from female C57BL/129 mice in accordance with IACUC protocol. RBCs were pelleted at 800 g for 30 min and resuspended and washed twice with a 40 mM-HEPES buffer (pH=7.4, 5 mM glucose, Sigma, St. Louis, MO; tonicity, 285 mOsm regulated with NaCl). The RBC suspensions were prepared at a concentration of 2×10^6 cells/mL. ([Hb]=0.93 μ M, [heme]=3.72 μ M) in the same HEPES buffer.

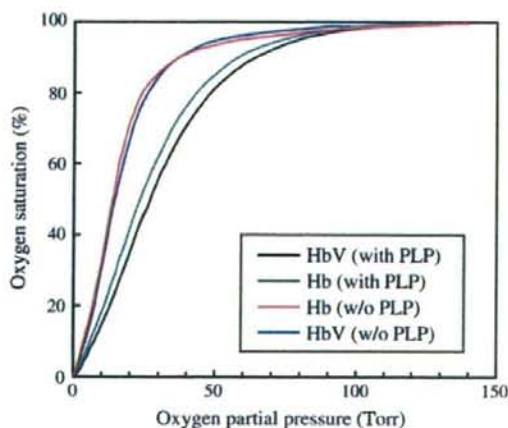


Fig. 1. Oxygen dissociation curves of Hb solutions and HbV with or without PLP measured with Hemox-Analyzer at 37 °C. The P₅₀ values and Hill numbers are listed in Table 1. The data of HbV (w/o PLP) is cited from our previous study [23].

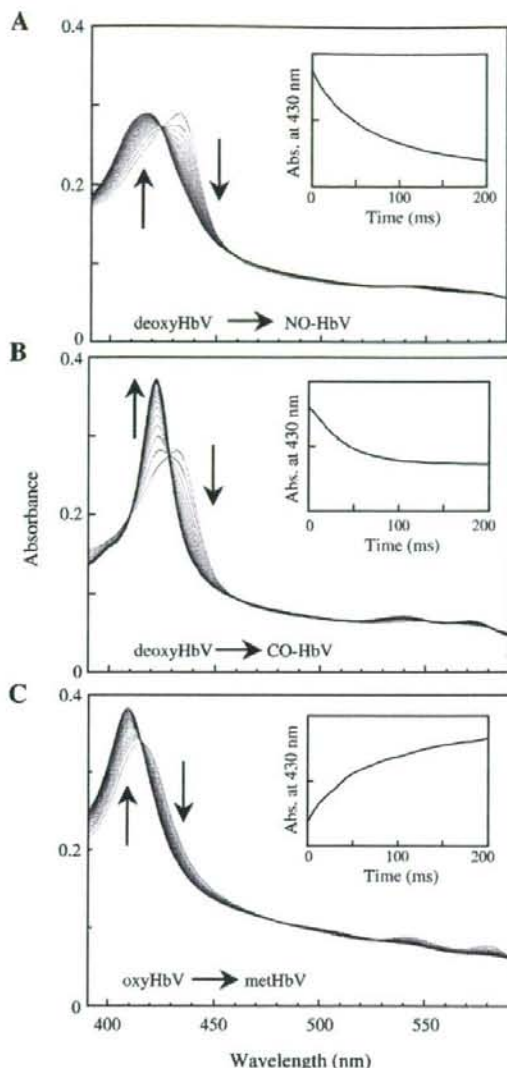


Fig. 2. Representative profiles of the reactions of NO or CO-HbV using stopped-flow spectrophotometry. (A) A NO-bubbled PBS ([NO]=3.8 μ M) and deoxygenated HbV in PBS ([heme]=3.0 μ M) were mixed rapidly; the absorption spectra were collected every millisecond for 0.2 s after mixing. In this figure, the spectroscopic curves of every 10 ms are selected. The figure shows clearly that the spectrum of deoxyHbV is mostly converted to NO-HbV during 0.2 s. (inset) The time course of the measured absorbance at 430 nm. (B) A CO-bubbled PBS ([CO]=135 μ M) and deoxygenated HbV in PBS ([heme]=3.0 μ M) were mixed rapidly using a stopped-flow spectrophotometer. This figure clearly shows that the spectrum of deoxyHbV is mostly converted to CO-HbV in 0.2 s. (inset) The time course of the measured absorbance at 430 nm. (C) A NO-bubbled PBS ([NO]=3.8 μ M) and oxygenated HbV in PBS ([heme]=3.0 μ M) were mixed. The spectrum of oxygenated HbV is mostly converted to metHbV during 0.2 s. (inset) The time course of the measured absorbance at 408 nm. The optical path length was 1 cm. In these experimental conditions, the CO binding in (C) seems faster than the NO reactions (A and B), due to the higher concentration of CO (135 μ M) than that of NO (3.8 μ M). However, the calculated reaction rate constant of the CO binding is much smaller than those of NO reactions as shown in Table 1.

2.2. Stopped-flow spectrophotometry

The time course of the ligand binding was analyzed during rapid mixing of the deoxygenated HbV and Hb solutions and a NO- or CO-containing solution using a stopped-flow rapid scan spectrophotometer (RSP-1000; Unisoku Co. Ltd., Osaka, Japan)

[22,23]. Solutions in the two gas-tight reservoirs (A and B) are mixed rapidly applying a pressure of 0.3–0.6 MPa; the dead time for mixing being <1.5 ms. All measurements were performed at 25 °C. A PBS solution (3 ml each) was poured into both reservoirs, which were both sealed using septum rubber seals. The reservoirs were deoxygenated by N_2 bubbling for more than 30 min. A stock solution of HbV or Hb (ca. 30 μ l, [heme]=300 μ M) was injected into Reservoir A to adjust [heme] to 3 μ M and the N_2 bubbling was changed to a flow to avoid denaturation of the solutes. Complete deoxygenation was confirmed using preliminary stopped-flow measurements (wavelength: 385–593 nm), where the Soret band showed a maximum absorbance (λ_{max}) at 430 nm because of the presence of deoxyHb. To make the reservoirs deoxygenated, we did not use sodium dithionite because it is well known that an excess amount of this reagent sometimes possibly induces chemical modifications of heme or globin [32,33]. In Reservoir B, NO or CO gas bubbling was started while a gentle N_2 flow was maintained in Reservoir A. The mixed gases for NO binding (NO, 0.2029%; N_2 , 99.7971%) and for CO binding (CO, 14.14%; N_2 , 85.86%) were purchased from Takachiho Chemical Industrial Co., Ltd. (Tokyo).

Stopped-flow measurement was initiated after about 10 min bubbling. The sampling interval and the exposure time were set as 1 ms and measurement time was 210 ms. All measurements were performed 3 times and the change of absorbance at 430 nm was plotted as a function of time. The apparent binding rate constants of NO and CO ($k_{on}^{(NO)}$ and $k_{on}^{(CO)}$, respectively) were calculated using Eq. (1) under the assumption of homogeneous distribution of Hb, irreversible second order reaction, and a pseudo-first order reaction when gas molecules are abundant.

$$\ln \frac{\Delta A_t}{\Delta A_0} = -k_{on}^{(G)} \cdot C_{G_0} \cdot t \quad (1)$$

In this equation, ΔA_t is the change of absorbance at 430 nm at time t ($=A_t - A_{t \rightarrow \infty}$) and A_0 is the absorbance at the initial time ($=A_{t=0} - A_{t \rightarrow \infty}$), and C_{G_0} is the initial gas concentration. NO (1.9 μ M) was not much more abundant than the heme concentration (1.5 μ M) in the NO-reaction experiments, therefore the apparent binding rates were calculated from the slopes of the initial phase of reactions. The time courses of the reaction of NO and O_2 -bound HbV or HbO₂ solutions were analyzed using stopped-flow spectrometry in the same manner, except that an air-equilibrated PBS solution was introduced into Reservoir A to ensure the O_2 -binding state. The absorption changes were monitored at 408 nm.

2.3. Measurement of NO depletion using a NO electrode

The rate of NO depletion in a test solution after adding NO in an aerobic condition was monitored via an amino-700 probe and an inNO-T sensor (Innovative Instruments, Inc.) connected to a Dell PC running InNO-T software (Innovative Instruments, Inc.) [34]. In a room atmosphere, 3 mL of a PBS solution containing HbV, EV, or mice RBCs was pipetted into a cuvette and stirred using a magnetic stir bar. The NO sensor was introduced into the cuvette for continuous monitoring. After a steady condition was confirmed, a stock NO solution (100 μ l) was injected, yielding the concentration of 2 μ M NO. The NO level rapidly increases and then decreases with the reaction with O_2 and Hb. Concentrations of the specimens were as follows: HbV ([heme]=4 μ M, [Hb]=65 mg/L, [Lipid]=36 mg/L), EV ([lipid]=36 mg/L), and mice RBC ([heme]=3.72 μ M). Data were

fitted and basic statistics and graphs were obtained using Synergy KaleidaGraph 3.6. Correlation was determined using Spearman's rank correlation test, with StatView 5.

3. Results

3.1. Stopped-flow spectrophotometry of NO and CO bindings in an anaerobic condition

Complete deoxygenation of HbV was confirmed using the characteristic wavelength of the maximum absorption (λ_{max}) at 430 nm. Because of the stronger light-scattering effect of the HbV suspension than that of the Hb solution and RBCs [35], the absorption peaks in the Q band region were not well defined in this measurement. After rapid mixing with NO, the immediate absorption reduction at 430 nm and the increase at 418 nm that correspond to the formation of HbNO were confirmed (Fig. 2A). In the case of mixing with CO, the immediate absorption increase at 419 nm was confirmed (Fig. 2B). The change of absorption at 430 nm in both reactions was indicative of the formation of HbNO or HbCO in the vesicles.

The spectrophotometric scans presented in Fig. 2A and B were performed 3 times and the averaged level of reaction was plotted as a ratio of absorbance at 430 nm (A_t) at time t , to the initial absorbance (ΔA_0) at time 0 (Fig. 3A and B). Data of Hb solutions are also plotted in those figures. The graph clearly shows that both NO binding and CO binding were slower for HbV than for the human Hb solution without PLP. However, Hb with PLP showed almost identical CO binding to HbV. The NO binding rates of Hb solution with and without PLP and poly₈Hb were almost identical. The apparent binding rate constants of $k_{on}^{(NO)}$ for NO and $k_{on}^{(CO)}$ for CO are presented in Table 1.

3.2. Stopped-flow spectrophotometry of the reaction of NO and O_2 -bound HbV and Hb solutions in an aerobic condition

The oxygenated HbV showed the absorption maximum at 415 nm (Fig. 2C). Mixing with NO showed the immediate reduction of the absorption maximum, although an increase at 405 nm showed attributes of the metHb formation. The spectrophotometry scans portrayed in Fig. 2C were performed 3 times and the average level of reaction was plotted as a ratio of absorbance at 408 nm ($\Delta A_t = A_t - A_{t \rightarrow \infty}$) at time t , to the initial absorbance ($\Delta A_0 = A_{t=0} - A_{t \rightarrow \infty}$) at time 0 (Fig. 3C).

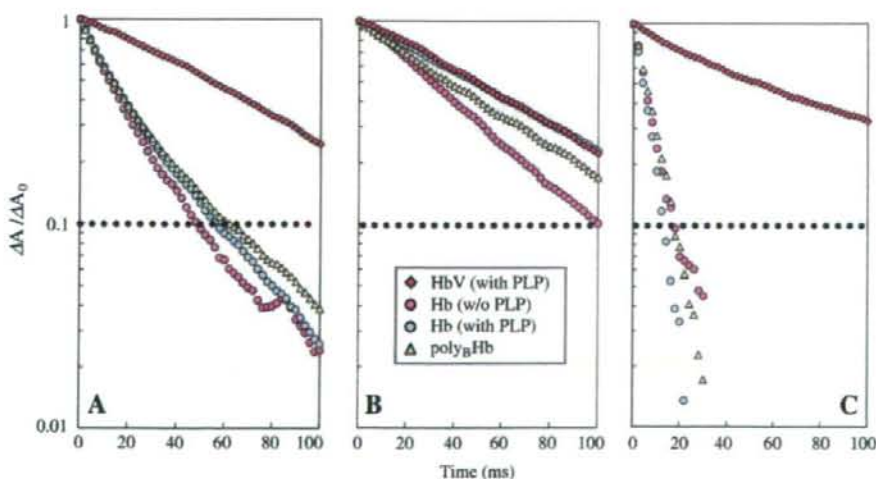


Fig. 3. Time courses of NO and CO reactions by HbV, human Hb solutions, and Poly₈Hb. The level of reaction was plotted on a semi-logarithmic graph as a ratio of absorbance at 419 or 430 nm (ΔA_t) at time t , to the initial absorbance (A_0) at time 0. (A) A NO-bubbled PBS ([NO]=3.8 μ M) and deoxygenated-Hb-containing solutions in PBS ([heme]=3.0 μ M) were mixed. (B) A CO-bubbled PBS ([CO]=135 μ M) and deoxygenated-Hb-containing solutions in PBS ([heme]=3.0 μ M) were mixed. (C) A NO-bubbled PBS ([NO]=3.8 μ M) and an oxygenated-HbO₂-containing solution in PBS ([heme]=3.0 μ M) were mixed.

Table 1

Apparent reaction rate constants of Hb, HbV, and polyHb with different P_{50} . The data of HbV without PLP were referred from a previous paper [23]

	HbV (with PLP)	HbV ^a (w/o PLP)	Hb (with PLP)	Hb (w/o PLP)	polyHb
$k_{on}^{(CO)}$ ($10^5 M^{-1} s^{-1}$)	2.1	3.4 ^a	2.1	3.4	2.7
$k_{off}^{(NO)}$ ($10^7 M^{-1} s^{-1}$)	0.61	0.88 ^a	2.4	2.6	2.4
$k_{off}^{(NO)}$ ($10^7 M^{-1} s^{-1}$)	0.88	–	8.7	7.4	6.6
P_{50} (Torr)	25–31	14 ^a	23	14	54 ^b
Hill number	2.1	2.1	1.9	2.2	1.2 ^b
Size	279 nm	265 nm ^a	65 kDa	65 kDa	87–502 kDa ^c

^a Reference [23].

^b Reference [31].

^c Reference [25].

The graph shows that the reaction of NO with oxygenated HbV was considerably slower than that of human Hb solutions with and without PLP. The rate of polyHb was almost identical to that of human Hb solutions. The apparent oxidation reaction rate constants $k^{(NO)}$ are summarized in Table 1.

3.3. NO depletion in an aerobic condition in the presence of HbV, RBC, and EV

When the stock NO solution was simply injected into a PBS solution, the NO electrode showed an immediate increase to about 2000 nM, even in the aerobic condition. Then it gradually decreased according to the reaction of NO and O_2 to produce NO_2 (Fig. 4). From the slope after 200 s, the apparent decay constant is calculated as

$3.7 \times 10^{-3} s^{-1}$. In the case of the presence of EV, the increase in NO concentration immediately after injection and the decay curve were almost identical, and the apparent decay constant was $3.9 \times 10^{-3} s^{-1}$. From results of this experiment, it is not clear that the hydrophobic part of the lipid bilayer membrane facilitates NO depletion. The reactions of NO in the aqueous phase are still the prominent ones.

In the presence of HbV, the first injection of NO (2000 nM) was decayed much faster than the sensor was able to record, and only a part of the second NO injection was recorded. The decay curve was finally obtained at the third NO injection when the heme of HbV was totally inactivated with NO and converted to the ferric state. On the other hand, one injection of NO into the RBC suspension clearly showed the decay curve. However, the rate of decay is extremely fast, as indicated by the need for a different time scale of the abscissa. The NO decay constant of HbV was not obtained from this experiment, which has to be analyzed by stopped-flow spectrophotometry.

4. Discussion

Our primary finding is that NO binding is considerably retarded by encapsulation into phospholipid vesicles, and that CO binding is not influenced by encapsulation, but is slightly retarded by co-encapsulation with PLP as an allosteric effector. In the aerobic condition, the hydrophobic part of the lipid bilayer membrane does not seem to be involved in NO depletion (facilitated reaction with O_2), which is mostly attributable to the reaction with the intracellular Hb solution.

We recently showed that NO binding of Hb is retarded by encapsulation in phospholipid vesicles because of the intracellular diffusion barrier [23]. Intrinsically rapid NO binding causes Hb to become

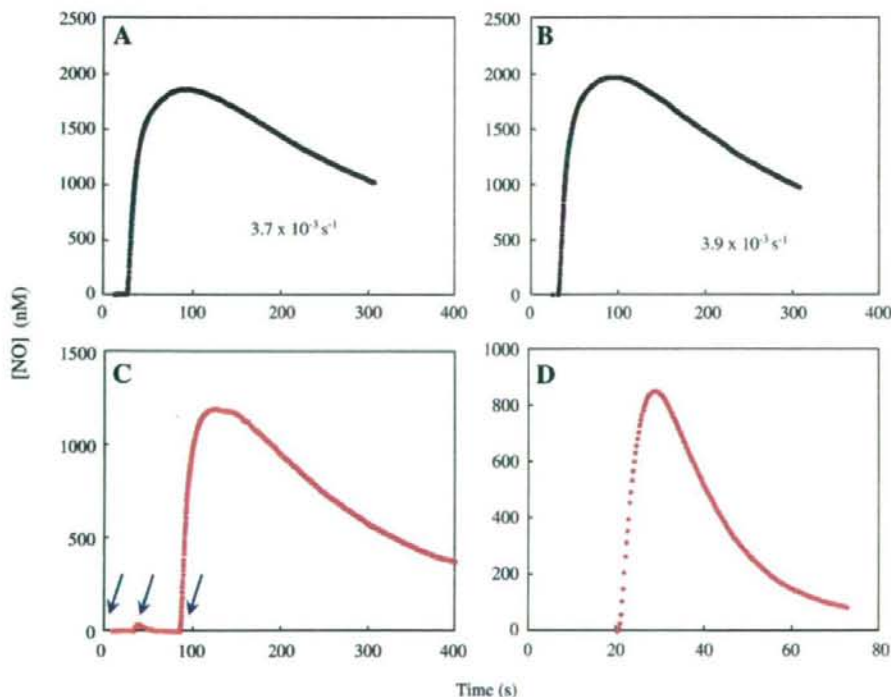


Fig. 4. NO decay in an aerobic condition in PBS solution after injection of NO yielding a concentration of 2000 nM NO. (A) PBS solution, as a standard curve of NO decay by the dissolved O_2 . The decay rate is calculated as $3.7 \times 10^{-3} s^{-1}$. (B) PBS solution containing EV ([lipids]=36 mg/mL). The decay rate is calculated as $3.9 \times 10^{-3} s^{-1}$. (C) PBS solution containing HbV ([heme]=4 μM , [Hb]=65 mg/L, [Lipid]=36 mg/L). The arrows indicate the points of three injections. At the first and the second injections, HbV consumed NO so rapidly that the NO electrode was undetectable. At the third injection, Hb of HbV would be totally inactivated, and the NO level increased, and subsequently decreased gradually through reaction with the physically-dissolved O_2 . (D) A PBS solution containing mice RBCs ([heme]=3.72 μM). In contrast to HbV, the NO level increased at the first NO injection, although it did not reach to 2000 nM, and the NO decay was much faster than that in (A).

a sink of NO in the interior surface region of HbV, thereby hindering further NO diffusion into the core of HbV, in combination with the lowered diffusion constants of NO in a highly concentrated Hb solution. Using results described in a previous report, we compare two kinds of HbV with different P_{50} values (25 and 14 Torr), and showed that the $k_{on}^{(NO)}$ might change slightly according to P_{50} (0.61×10^7 vs. $0.88 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). Accordingly, the major cause of retardation is Hb encapsulation. Cell-free Hb removes NO much faster (cf. $k_{on}^{(NO)}$ of cell-free Hb solution, $2.4\text{--}2.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). Cell-free Hb solutions with and without PLP did not differ greatly. The results coincide well with those in precedent reports: that NO binding to α and β subunits and that the T-state and R-state Hbs are almost identical [36,37]. Poly β Hb that contains high concentration of tetrameric β Hb and intermolecularly crosslinked tetrameric β Hb are known to induce vasoconstriction and hypertension [38]. In the present study, such Poly β Hb showed identical rapid NO binding.

For CO, we previously reported that CO binding to Hb is intrinsically much slower than NO binding by two orders of magnitude, which allows more time for CO diffusion into the HbV core. Therefore, $k_{on}^{(CO)}$ is not influenced by the intracellular Hb concentration and particle size smaller than 500 nm [23]. Results of the present study show the presence of a slight retardation of CO binding by HbV in comparison to the cell-free Hb solution, which is solely attributable to the co-encapsulation of PLP as an allosteric effector. The effector tends to stabilize the T-state and retards CO binding. In contrast to NO, it is well known that cooperative CO binding to four subunits of Hb occurs, and that the binding rate constants to α and β subunits, or T-state and R-state Hbs, are different [39,40]. According to Kwansa et al. [41], the value of $k_{on}^{(CO)}$ of unmodified β Hb measured using stopped-flow is $1.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Polymerization of β Hb by glutaraldehyde apparently facilitates CO binding ($k_{on}^{(CO)}$ of Poly β Hb = $2.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$).

In the circulation where O_2 is abundant NO is inactivated mainly by NO dioxygenation by O_2 -bound HBOCs and RBC. Results of the present study confirmed that the cell-free human Hb solutions with or without PLP showed $k_{on}^{(NO)}$ of $7.4\text{--}8.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, which was faster than that of the reaction of deoxyHb and NO ($k_{on}^{(NO)}$ = $2.4\text{--}2.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), a tendency that coincided with the results of Herold et al. [42]. Encapsulation of HbO₂ in vesicles significantly retarded the reaction with NO, and $k_{on}^{(NO)}$ was $0.88 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. However, we observed the change of heme ligation state (HbO₂ → metHb) and we were unable to observe the NO concentration using stopped-flow spectrophotometry. In an aerobic condition, NO reacts slowly with O_2 to produce NO₂ in water.

The weight ratio of lipids to Hb of HbVs is about 0.5–0.6, this ratio being as low as 0.15 for RBCs. We anticipated that the hydrophobic part of the lipid bilayer membrane of HbV would be involved considerably in NO depletion, as reported by Liu et al. [24], which might affect the results of stopped-flow spectrophotometry. We monitored the NO decay in the presence of empty vesicles (without Hb) in an aerobic condition using a NO electrode to clarify the facilitation of NO depletion. Results show that NO decay in the presence of empty vesicles was almost identical to that in the PBS solution, indicating that the facilitated reaction of NO and O_2 in a hydrophobic part of the lipid bilayer membrane is negligibly small compared to the reaction with the intracellular Hb.

This finding may be related to the lipid composition of the membrane. The saturated phospholipid (DPPC, phase transition temperature = 41 °C) with cholesterol in HbV might be more resistant to oxidative damage than is soy phosphatidylcholine reported by Liu et al. [24], which probably contains many unsaturated fatty acids. Moreover, nitration of unsaturated bonds might occur, which is another pathway for NO depletion [43]. Conversely, our lipid mixture of DPPC comprises saturated fatty acids and cholesterol providing a hydrophobic rigid domain that would differ from the liposomes made of soy phospholipids. If the aqueous reaction of NO and dioxygen occurs with an overall stoichiometry of $4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-$ [24,44], then H_2O is required even in the hydrophobic part. The unstable soy

phospholipid vesicles in the literatures would provide more water molecules than does the rigid and stable bilayer membrane of our HbV. Accordingly, we conclude that NO depletion in aerobic conditions in the presence of HbV is caused primarily by the encapsulated Hbs, and the contribution of the bilayer membrane of HbV is negligibly small.

The NO decay in HbV was much faster than that for RBCs. In addition, measurements using the NO electrode were unable to detect the reaction rate of O_2 -HbV and NO. These results demonstrate that NO mainly reacts with Hb in HbV. Given the rate of NO decay shown in Fig. 4, we can assume that less than 0.1% of total NO (1.9 μM) reacts with the physically-dissolved O_2 in PBS in 200 ms after the rapid mixing by stopped-flow spectrophotometry.

In a previous report on the relative magnitude of the binding rate constant of NO (fast) and CO (slow) to deoxygenated HbV, we predicted that the strength of the diffusion barrier induced in HbV becomes more significant with the faster ligand binding reaction with Hb [23]. According to our results and Herold et al. [42], the elementary reaction of NO and HbO₂ is much faster than that of NO with deoxyHb. As shown in Fig. 2, Hb encapsulation in vesicles retarded NO binding to deoxyHb by 1/4, and NO reaction with HbO₂ by 1/8. The impact of Hb encapsulation on the reaction with NO is strengthened in the aerobic condition.

We previously reported that HbV does not induce hypertension. However, present results, which show retardations of NO binding by encapsulation and CO binding by co-encapsulation of PLP in comparison to cell-free Hb solutions and poly β Hb, cannot fully explain the absence of vasoconstriction or hypertension after intravenous injection because these reactions of HbV are much greater than those of RBCs ($10^4\text{--}10^5 \text{ M}^{-1} \text{ s}^{-1}$) [1,2,37,45]. Any Hb-based oxygen carrier (HBOC) is much smaller than an RBC and is distributed homogeneously in the plasma layer [46]. Consequently, an RBC-free zone at the blood/endothelium interface becomes an HBOC-containing zone, and might be a sink of NO [47,48]. Rohlf's et al. [49] reported that NO binding rate constants of a series of chemically modified HBOCs (diameter, 6–28 nm), measured using the laser flash photolysis, were identical to that of an unmodified Hb solution: $3.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. They concluded straightforwardly that NO uptake was unrelated to vasoconstriction because PEG-modified Hb did not induce vasoconstriction, other mechanisms being suggested as determinants of vasoconstriction such as molecular recognition, oxygen affinity [7,50] and facilitated diffusion. Poly β Hb is larger than monomeric β Hb, but may be vasoactive because of the presence of monomeric β Hb and its high P_{50} [25,31].

It is also speculated that there is a threshold particle diameter to penetrate across the perforated endothelial cell layer to approach a space (such as the space of Disse near the sinusoidal endothelial layer

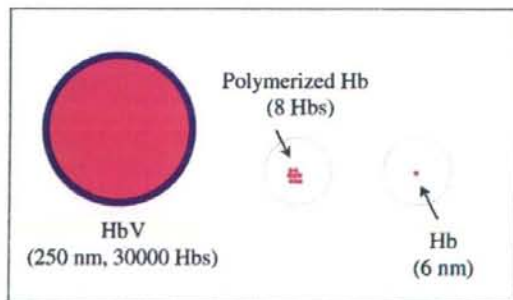


Fig. 5. Comparison of particle size between HbV (250 nm), Hb (6 nm) and polymerized Hb. According to the molecular weight distribution of Poly β Hb [31], the molecular weight (Mw) of the largest polymerized Hb is 502 kDa, which corresponds to 8 Hbs. One HbV contains about 30,000 Hbs. It is obvious that HbV is much larger than the largest fraction of Poly β Hb. For the retardation of NO binding, both Hb concentration in the particle and the particle size are important [23].

in a hepatic microcirculation, or the space between the endothelium and the smooth muscle), where CO or NO is produced as a vaso-relaxation factor. Because the particle size of HbV (250 nm) is obviously much larger than the Hb tetramer and its polymerized form as shown in Fig. 5 both the retardation of NO reaction and the larger particle diameter are inferred to be keys to suppress vasoconstriction and hypertension induced by HBOCs.

In conclusion, the source of vasoactivity of HBOCs has been extensively reviewed [7,9,13,38,51–54] and it is generally agreed that NO scavenging is a factor in this process, although it does not provide an all encompassing explanation. In terms of HbV, the intrinsically fast reactions of NO with the cell-free Hb solutions in both aerobic and anaerobic conditions are considerably retarded by the encapsulation process. Therefore, since NO bioavailability results from the balance of the rate of NO production and NO entrapment or scavenging, the finding that the latter processes are significantly retarded in HbVs provides some meaningful information to explain their vasoactivity.

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References

- Carlsen, J.H., Comroe, The rate of uptake of carbon monoxide and of nitric oxide by normal human erythrocytes and experimentally produced spherocytes. *J. Gen. Physiol.* 42 (1958) 83–107.
- Liu, M.J., Miller, M.S., Joshi, H., Sadowska-Krowicka, D.A., Clark, J.R., Lancaster Jr., Diffusion-limited reaction of free nitric oxide with erythrocytes. *J. Biol. Chem.* 273 (1998) 18709–18713.
- M.W. Vaughn, K.T. Huang, L. Kuo, J.C. Liao, Erythrocytes possess an intrinsic barrier to nitric oxide consumption. *J. Biol. Chem.* 275 (2000) 2342–2348.
- P.C. Minneci, K.J. Deans, H. Zhi, P.S. Yuen, R.A. Star, S.M. Banks, A.N. Schechter, C. Natanson, M.T. Gladwin, S.B. Solomon, Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by compartmentalized oxyhemoglobin. *J. Clin. Invest.* 115 (2005) 3409–3417.
- J.R. Hess, V.W. MacDonald, W.W. Brinkley, Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. *J. Appl. Physiol.* 74 (1993) 1769–1778.
- E.P. Sloan, M. Koenigsberg, D. Gens, M. Cipolle, J. Runge, M.N. Mallory, G. Rodman Jr., Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial. *JAMA* 282 (1999) 1857–1864.
- A. Gulati, A. Barve, A.P. Sen, Pharmacology of hemoglobin therapeutics. *J. Lab. Clin. Med.* 133 (1999) 112–119.
- G. Rochon, A. Caron, M. Toussaint-Hacquad, A.I. Alayash, M. Gentils, P. Labrude, J.F. Stoltz, P. Menu, Hemodilution with stroma-free hemoglobin at physiologically maintained viscosity delays the onset of vasoconstriction. *Hypertension* 43 (2004) 1110–1115.
- H. Sakai, H. Hara, M. Yuasa, A.G. Tsai, S. Takeoka, E. Tsuchida, M. Intaglietta, Molecular dimensions of Hb-based O₂ carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 279 (2000) H908–H915.
- K. Nakai, T. Ohta, I. Sakuma, K. Akama, Y. Kobayashi, S. Tokuyama, A. Kitabatake, Y. Nakazato, T.A. Takahashi, S. Sekiguchi, Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. *J. Cardiovasc. Pharmacol.* 28 (1996) 115–123.
- J.S. Olson, E.W. Foley, C. Rogge, A.L. Tsai, M.P. Doyle, D.D. Lemon, NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic. Biol. Med.* 36 (2004) 685–697.
- M. Suematsu, N. Goda, T. Sano, S. Kashiwagi, T. Egawa, Y. Shinoda, Y. Ishimura, Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J. Clin. Invest.* 96 (1995) 2431–2437.
- N. Goda, K. Suzuki, M. Naito, S. Takeoka, E. Tsuchida, Y. Ishimura, T. Tamatani, M. Suematsu, Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J. Clin. Invest.* 101 (1998) 604–612.
- X. Liu, A. Samouilov, J.R. Lancaster Jr., J.L. Zweier, Nitric oxide uptake by erythrocytes is primarily limited by extracellular diffusion not membrane resistance. *J. Biol. Chem.* 277 (2002) 26194–26199.
- D.B. Kim-Shapiro, A.N. Schechter, M.T. Gladwin, Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler. Thromb. Vasc. Biol.* 26 (2006) 697–705.
- K.T. Huang, Z. Huang, D.B. Kim-Shapiro, Nitric oxide red blood cell membrane permeability at high and low oxygen tension. *Nitric Oxide* 16 (2007) 209–216.
- K.T. Huang, T.H. Han, D.R. Hyde, M.W. Vaughn, H. Van Herle, T.W. Hein, C. Zhang, L. Kuo, J.C. Liao, Modulation of nitric oxide bioavailability by erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 11771–11776.
- T.H. Han, A. Pelling, T.J. Jeon, J.K. Gimzewski, J.C. Liao, Erythrocyte nitric oxide transport reduced by a submembrane cytoskeletal barrier. *Biochim. Biophys. Acta* 1723 (2005) 135–142.
- J.T. Cohn, J.S. Olson, The rate of oxygen uptake by human red blood cells. *J. Biol. Chem.* 254 (1979) 1178–1190.
- L. Djordjevic, I.F. Miller, Synthetic erythrocytes from lipid encapsulated hemoglobin. *Exp. Hematol.* 8 (1980) 584–592.
- E. Tsuchida, H. Sakai, H. Horinouchi, K. Kobayashi, Hemoglobin-vesicles as a transfusion alternative. *Artif. Cells Blood Substit. Biotechnol.* 34 (2006) 581–588.
- H. Sakai, K. Hamada, S. Takeoka, H. Nishide, E. Tsuchida, Functional evaluation of hemoglobin- and lipid-heme-vesicles as red cell substitutes. *Polymer Adv. Technol.* 7 (1996) 639–644.
- H. Sakai, A. Sato, K. Masuda, S. Takeoka, E. Tsuchida, Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J. Biol. Chem.* 283 (2008) 1508–1517.
- X. Liu, M.J. Miller, M.S. Joshi, D.D. Thomas, J.R. Lancaster Jr., Accelerated reaction of nitric oxide with O₂ within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 2175–2179.
- P.W. Buehler, R.A. Boylkins, Y. Jia, S. Norris, D.I. Freedberg, A.I. Alayash, Structural and functional characterization of glutaraldehyde-polymerized bovine hemoglobin and its isolated fractions. *Anal. Chem.* 77 (2005) 3466–3478.
- H. Sakai, S. Takeoka, H. Yokohama, Y. Seino, H. Nishide, E. Tsuchida, Purification of concentrated hemoglobin using organic solvent and heat treatment. *Protein Expr. Purif.* 4 (1993) 563–569.
- H. Sakai, Y. Masada, S. Takeoka, E. Tsuchida, Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. *J. Biochem. (Tokyo)* 131 (2002) 611–617.
- S. Takeoka, T. Ohgushi, K. Terase, T. Ohmori, E. Tsuchida, Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir* 12 (1996) 1755–1759.
- K. Sou, Y. Naito, T. Endo, S. Takeoka, E. Tsuchida, Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol. Prog.* 19 (2003) 1547–1552.
- L. Wang, K. Morizawa, S. Tokuyama, T. Satoh, E. Tsuchida, Modulation of oxygen-carrying capacity of artificial red cells. *Polymers Adv. Technol.* 4 (1993) 8–11.
- P. Cabrales, A.G. Tsai, M. Intaglietta, Increased tissue PO₂ and decreased O₂ delivery and consumption after 80% exchange transfusion with polymerized hemoglobin. *Am. J. Physiol. Heart Circ. Physiol.* 287 (2004) H2825–H2833.
- J. Herzfeld, N.E. Seidel, M.P. Taylor, P.R. Droupadi, N.E. Wang, Gentle chemical deoxygenation of hemoglobin solutions. *Hemoglobin* 14 (1990) 399–411.
- C.A. Sawicki, Q.H. Gibson, Tetramer-dimer dissociation of carboxyhemoglobin in the absence of dithionite. *Biophys. J.* 35 (1981) 265–270.
- I. Gramaglia, P. Sobolewski, D. Meays, R. Contreras, J.P. Nolan, J.A. Frangos, M. Intaglietta, H.C. van der Heyde, Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. *Nat. Med.* 12 (2006) 1417–1422.
- H. Sakai, K. Tomiyama, Y. Masada, S. Takeoka, H. Horinouchi, K. Kobayashi, E. Tsuchida, Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. *Clin. Chem. Lab. Med.* 41 (2003) 222–231.
- R. Cassoly, Q. Gibson, Conformation, co-operativity and ligand binding in human hemoglobin. *J. Mol. Biol.* 91 (1975) 301–313.
- J.S. Olson, Stopped-flow, rapid mixing measurements of ligand binding to hemoglobin and red cells. *Methods Enzymol.* 76 (1981) 631–651.
- P. Pawson, I.F. Gibson, F.J. Dowell, The effect of the polymerized bovine haemoglobin solution, Hb-200, on endothelial function in isolated arterial rings from rats. *J. Vet. Pharmacol. Therap.* 30 (2007) 556–563.
- M. Brunori, J. Bonaventura, C. Bonaventura, E. Antonini, J. Wyman, Carbon monoxide binding by hemoglobin and myoglobin under photodissociating conditions. *Proc. Natl. Acad. Sci. U. S. A.* 69 (1972) 868–871.
- M. Perrella, N. Davids, L. Rossi-Bernardi, The association reaction between hemoglobin and carbon monoxide as studied by the isolation of the intermediates. Implications on the mechanism of cooperativity. *J. Biol. Chem.* 267 (1992) 8744–8751.
- H.E. Kwansa, A.D. Young, D. Arosio, A. Razymska, E. Bucci, Adipyl crosslinked bovine hemoglobins as new models of allosteric systems. *Proteins* 39 (2000) 166–169.
- S. Herold, M. Exner, T. Nausner, Kinetic and mechanistic studies of the NO⁺-mediated oxidation of oxyhemoglobin and oxyhemoglobin. *Biochemistry* 40 (2001) 3385–3395.
- M.R. Baker, Y. Lin, F.J. Schopfer, S.R. Woodcock, A.L. Groeger, C. Bathany, S. Sweeney, M.H. Long, K.E. Iles, L.M. Baker, B.P. Brnchaud, Y.E. Chen, B.A. Freeman, Fatty acid transduction of nitric oxide signaling: multiple nitrate unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. *J. Biol. Chem.* 280 (2005) 42464–42475.
- P.C. Ford, D.A. Wink, D.M. Stanbury, Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* 326 (1993) 1–3.
- I. Azarov, K.T. Huang, S. Basu, M.T. Gladwin, N. Hogg, D.B. Kim-Shapiro, Nitric oxide scavenging by red blood cells as a function of hematocrit and oxygenation. *J. Biol. Chem.* 280 (2005) 39024–39032.
- H. Sakai, Y. Suzuki, M. Kinoshita, S. Takeoka, N. Maeda, E. Tsuchida, O₂ release from Hb vesicles evaluated using an artificial, narrow O₂-permeable tube: comparison with RBCs and acellular Hbs. *Am. J. Physiol. Heart Circ. Physiol.* 285 (2003) H2543–H2555.

- [47] M. Kavdia, N.M. Tsoukias, A.S. Popel, Model of nitric oxide diffusion in an arteriole: impact of hemoglobin-based blood substitutes, *Am. J. Physiol. Heart Circ. Physiol.* 282 (2002) H2245–H2253.
- [48] A. Jeffers, M.T. Gladwin, D.B. Kim-Shapiro, Computation of plasma hemoglobin nitric oxide scavenging in hemolytic anemias, *Free Radic. Biol. Med.* 41 (2006) 1557–1565.
- [49] R.J. Rohlf, E. Bruner, A. Chiu, A. Gonzales, M.L. Gonzales, D. Magde, M.D. Magde Jr., K.D. Vandegriff, R.M. Winslow, Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide, *J. Biol. Chem.* 273 (1998) 12128–12134.
- [50] A.C. Tsai, P. Cabrales, B.N. Manjula, S.A. Acharya, R.M. Winslow, M. Intaglietta, Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers, *Blood* 108 (2006) 3603–3610.
- [51] K. Nakai, I. Sakuma, T. Ohta, J. Ando, A. Kitabatake, Y. Nakazato, T.A. Takahashi, Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers, *J. Lab. Clin. Med.* 132 (1998) 313–319.
- [52] Y. Smani, A. Fiffre, P. Labrude, C. Vigneron, B. Faivre, Pharmacological and physicochemical factors in the pressor effects of conjugated haemoglobin-based oxygen carriers in vivo, *J. Hypertens.* 25 (2007) 599–608.
- [53] D.H. Doherty, M.P. Doyle, S.R. Curry, R.J. Vali, T.J. Fattor, J.S. Olson, D.D. Lemon, Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin, *Nat. Biotechnol.* 16 (1998) 672–676.
- [54] B. Matheson, H.E. Kwansa, E. Bucci, A. Rebel, R.C. Koehler, Vascular response to infusions of a nonextravasating hemoglobin polymer, *J. Appl. Physiol.* 93 (2002) 1479–1486.

The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection

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Verdu EF, Bercik P, Huang XX, Lu J, Al-Mutawaly N, Sakai H, Tompkins TA, Croitoru K, Tsuchida E, Perdue M, Collins SM. The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection. *Am J Physiol Gastrointest Liver Physiol* 295: G664–G670, 2008. First published July 24, 2008; doi:10.1152/ajpgi.90316.2008.—The role of chronic infections, such as *Helicobacter pylori* (*Hp*), to produce sustained changes in host physiology remains controversial. In this study, we investigate whether the antigenic or bacterial content of the gut, after *Hp* eradication, influences the changes in gut function induced by chronic *Hp* infection. Mice were infected with *Hp* for 4 mo and then treated with antibiotics or placebo for 2 wk. Gastric emptying was measured using videofluoroscopy, feeding behavior using a 24-h feeding system, and intestinal permeability using an isolated jejunal segment arterially perfused with an artificial oxygen carrier, hemoglobin vesicles. Immune responses were assessed by CD3⁺ cell counts and anti-*Hp* antibodies using ELISA. To determine the role of luminal factors in host physiology posteradication, groups of mice received the probiotics containing *Lactobacillus rhamnosus* R0011 and *L. helveticus* R0052 or placebo for 2 wk or crude *Hp* antigen weekly for 2 mo. Chronic *Hp* infection was associated with delayed gastric emptying, increased intestinal permeability, and increased gastric CD3⁺ cell counts. *Hp*-induced altered feeding patterns did not reverse after eradication. Probiotics accelerated the recovery of paracellular permeability and delayed gastric emptying, improved the CD3⁺ cell counts, and normalized altered feeding patterns posteradication. *Hp* antigen resulted in increased anti-*Hp* antibodies and increased CD3⁺ cell counts in the stomach and delayed recovery of gastric function. Our results suggest that the bacterial content of the gut, as well as the presence of relevant antigens, influences the rate of recovery of host pathophysiology induced by chronic *Hp* infection. These changes do not seem to occur in association with modulation of intestinal permeability.

lactobacilli; gastric emptying; feeding patterns

CONTROVERSY SURROUNDS THE ROLE of chronic *Helicobacter pylori* (*H. pylori*) infection as a cause of functional dyspepsia. Although it is known that *H. pylori* infection can alter gastric physiology, clinical evidence shows that there is little, if any, relief of symptoms following successful eradication of the bacterium in the short or medium term (4, 11, 16, 20, 21). The notion that acute bacterial infection can trigger a sequence of events resulting in chronic gastrointestinal symptomatology has now been established in the context of acute gastroenteritis as a trigger of both irritable bowel syndrome and functional dyspepsia (12, 17–19, 23). Less is known about the role of chronic infections, such as *H. pylori*, and their ability to

produce sustained changes in host physiology and possibly persistent symptoms.

Chronic gastric inflammation is the hallmark of *H. pylori* infection (7, 13). We have shown that, in mice, *H. pylori* infection induces functional and morphological changes in the gastric and spinal neural circuitry that are progressive and lymphocyte dependent (2). Some of these changes persist after bacterial eradication, suggesting that postinfective changes and immune activation are long lasting. In particular, altered feeding patterns, reminiscent of early satiety, are present up to 2 mo posteradication (posteradication) (3).

Persistence of symptoms may relate to luminal factors, which maintain low-grade inflammation after *H. pylori* eradication. Gut barrier function is crucial in limiting the effect of luminal antigens on the immune system. Thus, in this study, we examined whether the antigenic or bacterial content of the gut influences the changes in gastric function and feeding behavior induced by the *H. pylori* chronic infection. Specifically, we determined whether exposure to *H. pylori* antigen or probiotic bacteria influences host physiology.

Our results suggest that the bacterial content of the gut, as well as the presence of relevant antigens, influences the rate of recovery of host pathophysiology induced by *H. pylori* infection.

MATERIALS AND METHODS

Animals. Male BALB/c mice (Harlan, Indianapolis, IN) were purchased at the age of 6–8 wk and housed in a conventional specific pathogen-free unit at McMaster University Central Animal Facility. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Chronic *H. pylori* infection. Mice chronically infected with *H. pylori* Sydney strain for 4 mo ($n = 64$) and a group of uninfected controls were used ($n = 27$). Additional mice infected with *H. pylori* were used to monitor the establishment of a chronic infection ($n = 14$). Every 2 wk, beginning at 2 wk postinfection, 2 mice per group were euthanized, and *H. pylori* infection was verified using Warthin-Starry staining. Gastric emptying and 24-h feeding patterns were assessed at 4 mo of chronic infection. Additional mice were euthanized and used for ex vivo intestinal permeability measurements. *H. pylori* eradication therapy was administered thereafter using antibiotic-containing food pellets (Bio-Serv, Frenchtown, NJ) for 2 wk. Gastric emptying was reassessed 2 wk and 2 mo posteradication. Feeding patterns and intestinal permeability were reassessed 2 mo posteradication.

Inflammation. Stomach samples (Swiss rolls) were preserved in 10% formalin and then stained with hematoxylin and eosin (H & E).

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H & E-stained and Warthin-Starry-stained slides were examined under light microscopy to confirm *H. pylori* eradication after antimicrobial therapy and assess gastric inflammation. Mononuclear cell (MN) scores were graded in the corpus on a scale of 0–3 as described previously (2).

Immunostaining for CD3⁺ cells was performed on stomach paraffin sections using a modified method described previously (2). Rabbit anti-mouse CD3 (1:300; Dako, Glostrup, Denmark) was used as primary antibody followed by biotinylated swine anti-rabbit (1:300, Dako) and streptavidin peroxidase conjugate (1:600, Dako). The antibodies were visualized using 3-amino-9-ethylcarbazole and counterstaining with Mayers hematoxylin. Negative controls were performed in the absence of primary antibody. CD3⁺ cells were counted in two slides per mouse ($n = 12$ /group) and averaged. CD3⁺ cells present in three randomly selected fields in the corpus and antrum separately ($\times 63$, mucosa and submucosa) were counted. Samples from the jejunal loop were obtained at the end of each permeability experiment to test for tissue viability. Samples were fixed in 10% formalin, stained with H & E, and examined for tissue damage as a result of hypoxia using light microscopy. Gross villous architecture and the presence of cell desquamation and edema at villi tips were investigated.

H. pylori antibody measurement. Levels of anti-*H. pylori* IgG1 and IgG2A were measured at 2 mo posteradication by ELISA using biotinylated goat anti-mouse IgG2a and IgG1 (Southern Biotechnology Associates, Birmingham, AL) as described previously (9).

Gastric emptying. Mice were gavaged with 0.2 ml of 40% barium and placed in a custom-made restrainer. Videofluoroscopic images of stomach were taken at 0 and 4 min and stored for offline analysis using VCR (Panasonic). Video images were then digitized and analyzed using public domain NIH Image 1.62 software (developed at the U.S. National Institutes of Health). Gastric emptying was calculated by multiplying the area of stomach by mean optical density of the gastric area and was expressed as a percentage of barium expelled in 4 min.

Twenty-four-hour feeding patterns. Twenty-four-hour feeding patterns were assessed individually in mice placed in the separate cages. Food pellets were fastened on the feeding tray positioned 5 cm above the bottom of the cage, which was connected to the strain gauge placed above the cage. The weight of the feeding tray with food pellets was continuously recorded by computer. Data acquisition and analysis were performed using custom designed software (written by N. Al-Mutawaly). The number of feeding episodes, amount of food per episode, and total amount of food consumed was calculated. An eating bout was defined as an episode of food consumption lasting more than 20 s; two bouts were considered to be independent from each other if the interval of quiescence was longer than 5 min.

Intestinal permeability. Intestinal permeability *ex vivo* was investigated using an isolated arterially perfused jejunal loop (1, 22). Briefly, a 4-cm segment of the distal jejunum was selected, and a terminal branch of the superior mesenteric artery was cannulated with a polyethylene catheter under intraperitoneal ketamine/xylazine anesthesia. Tissue oxygenation was maintained by perfusion of the arterial branch with hemoglobin vesicles (Waseda University, Tokyo, Japan) (17a) using a peristaltic pump (Ismatec, Zurich, Switzerland). Luminal ends of the jejunal segment were cannulated using polyethylene cannulas. Following gentle washing of the lumen with saline to remove food residue, the jejunal loop was dissected and transferred to a custom-built organ chamber containing saline at 37°C. The loop was then perfused intraluminally with saline at 5 ml/h by using a syringe infusion pump (Harvard University, Boston, MA). All preparations were allowed to equilibrate for 5 min before collection of venous outflow, which was sampled continuously for the entire duration of the experiment (36 min) in 3-min fractions. Intestinal segment was perfused luminally

with isosmotic mixture of ⁵¹Cr-EDTA (0.6 μ Ci/ml) and ¹⁴C-mannitol (0.1 μ Ci/ml) (Perkin Elmer, Boston, MA) solution for 9 min at 5 ml/h. After the end of each experiment, two full-thickness tissue samples were excised from proximal and distal regions of the loop and fixed in formalin for later microscopic examination. Concentration of the radiolabeled macromolecules in the venous outflow was detected using a liquid scintillation Beta counter (LS 5801; Beckman Coulter, Mississauga, ON, Canada). The recovery of radioactivity in each venous outflow fraction is expressed as a proportion of that found in an identical volume of the luminal perfusate.

H. pylori antigen administration posteradication. To maintain persistence of gastric dysfunction posteradication a group of mice received crude *H. pylori* antigen (100 μ g/mouse) by gavage once weekly for 2 mo starting 1 wk after *H. pylori* eradication therapy. *H. pylori* antigen was prepared from fresh *H. pylori* cultures using liquid brain-heart infusion-based media as described previously (2). The bacteria were then gently centrifuged, and pellets were resuspended in saline and homogenized/disrupted with a sonicator. The concentration of bacterial antigen was adjusted with saline (100 μ l/mouse). Gastric emptying was reassessed at the end of *H. pylori* antigen administration (2 mo posteradication). Stomach samples were obtained at sacrifice and fixed in formalin for Warthin-Starry stain and for CD3⁺ cell counts.

Probiotic treatment posteradication. Groups of mice received by daily gavage either 100 μ l of placebo-maltodextrin dissolved in sterile water or 100 μ l of 10¹⁰ *Lactobacillus rhamnosus* (*L. rhamnosus*) R0011 and *L. helveticus* R0052 (Lacidofil) for 2 wk immediately after eradication therapy. Uninfected mice received maltodextrin treatment on a daily basis for 2 wk. Gastric emptying was reassessed at the end of probiotic treatment (2 wk posteradication) and at 2 mo posteradication. Fecal pellets were obtained at 2 wk to investigate probiotic survival in the gastrointestinal tract. Twenty-four-hour feeding patterns were reexamined at 2 mo posteradication. Additional mice were euthanized for *ex vivo* permeability measurements at 2 mo posteradication.

Detection of L. rhamnosus R0011 and L. helveticus R0052 in feces. Fresh fecal pellets were collected aseptically from the anal region into a sterile cryogenic tube containing 0.9% saline and 10% glycerol while the animals were kept in Plexiglas restrainers. For analysis, 100 μ l of fecal solution were pipetted into 25 ml of de Man, Rogosa, and Sharpe broth plus antibiotics (5.9 mg/ml phosphomycin and 18.6 mg/ml sulfamethoxazole) and 1 mg/ml in H₂SO₄ 0.02 M trimethoprim. Vancomycin (5 mg) was added for selective *L. rhamnosus* R0011 and ciprofloxacin (5 mg) for *L. helveticus* R0052 growth and was cultured anaerobically at 37°C for 48 h.

For DNA extraction and PCR amplification, 1.5 ml of each culture solution were centrifuged, and bacteria pellet was collected. Bacterial genomic DNA was extracted by Wizard Genomic DNA purification kit (Promega, Madison, WI) according to manufacturer's instructions. PCR amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer) as follows: in a 0.5 ml PCR tube, 1 μ l (0.2 μ g) of DNA was added to 49 μ l of PCR reaction mixture containing 200 μ M of each dNTP, 1.5 mM MgCl₂, 10 pmol amounts of each primer and 2.5 U of Taq DNA. PCR cycling parameters were 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C; after the last cycle, we added a final extension 7 min at 72°C. PCR product was visualized by ethidium bromide staining in 2% agarose gels. R0011 was used as a positive control, and *L. casei* R0215 were used as negative controls.

PCR primer pairs were as follows: *L. helveticus* R0052: sense 5'-ATTTTGGCAACT GTTACTCCATC-3', antisense 5'-GCATAAT-AGTCTTAGCTACGC-3'; *L. rhamnosus* R0011 (two pairs of primer sets): sense 5'-GACAATACTGATTTCCACC-3', antisense 5'-TC-C AATGTTCTCAACCAG-3' and sense TCAGTAGACACCTAC-CGG-3', antisense 5'-GTTGTAAGCTCTGGGACGCG. The first pair of primers can amplify *L. zeae* ATCC 393 and its prophage (ϕ AT3) due to the high homology between this phage and R0011

prophage, LarhR11-1. Therefore, only samples that were positive for both primer sets were considered positive for R0011.

Statistical analysis. Data are presented as means \pm SD or medians with interquartile ranges when appropriate. Data was analyzed using either two-way ANOVA, Kolmogorov-Smirnov test or non-paired *t*-test as appropriate. The Spearman rank correlation test was used to test the strength of association between parameters. A *P* value of <0.05 was considered statistically significant.

RESULTS

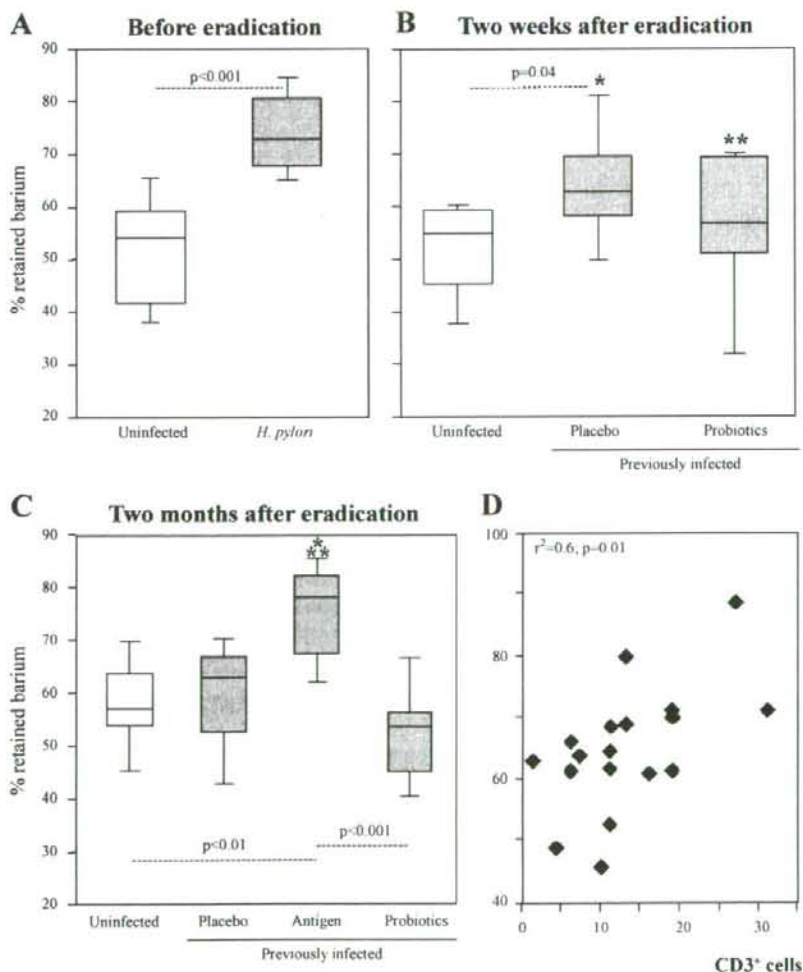
Effect of chronic *H. pylori* infection on inflammation. Chronic *H. pylori* infection (4 mo postinfection) induced chronic active inflammation, located mainly in the submucosal layer of the proximal stomach. The mononuclear cell score in the corpus was 0.6 ± 0.6 and 2.0 ± 0.7 ($P < 0.01$) in uninfected controls and *H. pylori*-infected mice, respectively. CD3⁺ cell scores in controls were 3.8 ± 3.0 and 0.8 ± 0.9 in corpus and antrum, respectively. During *H. pylori* infection, they increased to 14.3 ± 7.1 and 4.5 ± 1.2 (both $P < 0.01$ vs. uninfected controls).

Effect of chronic *H. pylori* infection on gastric emptying. In accordance with previous results (3), the percentage of retained barium during *H. pylori* infection was 30% higher than in uninfected mice (Fig. 1, before eradication). Gastric emptying fully normalized at 2 mo posteradication (Fig. 1, 2 mo after eradication).

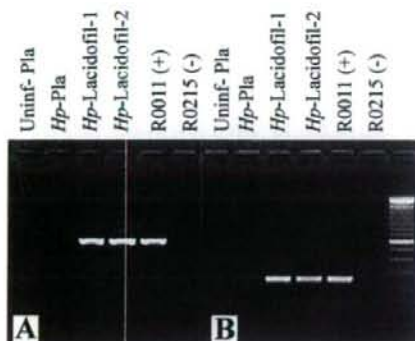
Effect of chronic *H. pylori* infection on body weight. In accordance with previous results (2, 3), there were no differences in body weight between uninfected and chronically *H. pylori*-infected mice (24.3 ± 2.6 g and 24.7 ± 3.0 g, respectively).

***H. pylori* antigen delays recovery of inflammation and gut function after bacterial eradication.** In mice previously infected with *H. pylori*, administration of crude *H. pylori* antigen maintained delayed gastric emptying for up to 2 mo posteradication (Fig. 1, 2 mo posteradication). Delayed gastric emptying correlated with increased CD3⁺ cell counts (Spearman rank correlation test, $P < 0.05$). Antigen-treated mice had IgG1 and IgG2A values nine- and 23-fold higher, respectively, compared with placebo-treated mice. Antigen administration did not affect gastric emptying in uninfected mice.

Fig. 1. A: gastric emptying was delayed in mice infected with *Helicobacter pylori* (*H. pylori*) ($n = 36$) compared with uninfected controls ($n = 20$). B: two weeks after eradication, gastric emptying improved in previously infected mice treated with placebo ($n = 12$, $*P = 0.02$ vs. *H. pylori*) but remained delayed compared with uninfected controls ($P = 0.04$). Previously infected mice treated with probiotics ($n = 12$) improved their gastric emptying ($**P < 0.01$ vs. *H. pylori*) and were similar to uninfected controls but were not different from placebo-treated mice. C: two months posteradication, gastric emptying normalized in placebo-treated ($n = 12$) mice but remained abnormal in antigen-treated mice ($n = 12$, $*P < 0.01$ vs. uninfected antigen, $**P = 0.02$ vs. uninfected placebo). D: degree of delayed gastric emptying was associated with the CD3⁺ cell counts in stomach ($r^2 = 0.6$; $P = 0.01$).



R0011



R0052



Fig. 2. Example of PCR detection of probiotics in feces. Two primers were used to detect R0011 (A and B) and one primer to detect R0052. Bands show negative detection of probiotics in feces of uninfected mice treated with maltodextrin (Uninf-Pla), infected mice treated with maltodextrin placebo (Hp-Pla), and negative control R0215. Positive detection is shown in 2 infected mice treated with probiotics (Hp-Lacidofil-1 and -2), and in positive controls using R0011 and R0052.

Detection of *L. rhamnosus* R0011 and *L. helveticus* R0052 in feces. Figure 2 shows an example of positive detection for *L. rhamnosus* R0011 and *L. helveticus* R0052 in feces at the end of probiotic feeding. All Lacidofil-fed mice tested positive for the specific probiotics in feces at the end of the probiotic administration period. No cross contamination was observed in mice gavaged with placebo.

Probiotics improve markers of inflammation posteradication. In placebo-treated mice, there was persistent infiltration with CD3⁺ T cells in the submucosal layer of the corpus and antrum 2

mo after eradication of *H. pylori*. In contrast, previously infected mice treated with probiotics exhibited a 60% lower MN score in corpus compared with placebo-treated mice (2.2 ± 0.5 vs. 0.8 ± 0.4 , $P = 0.01$). The CD3⁺ T cell infiltrate in both corpus and antrum was also reduced by probiotic therapy (Fig. 3). There was no overt inflammation in the jejunal segments of *H. pylori*-infected mice compared with uninfected controls.

Effect of probiotics on gastric emptying after *H. pylori* eradication. At 2 wk posteradication, previously *H. pylori*-infected mice treated with probiotics had returned to uninfected

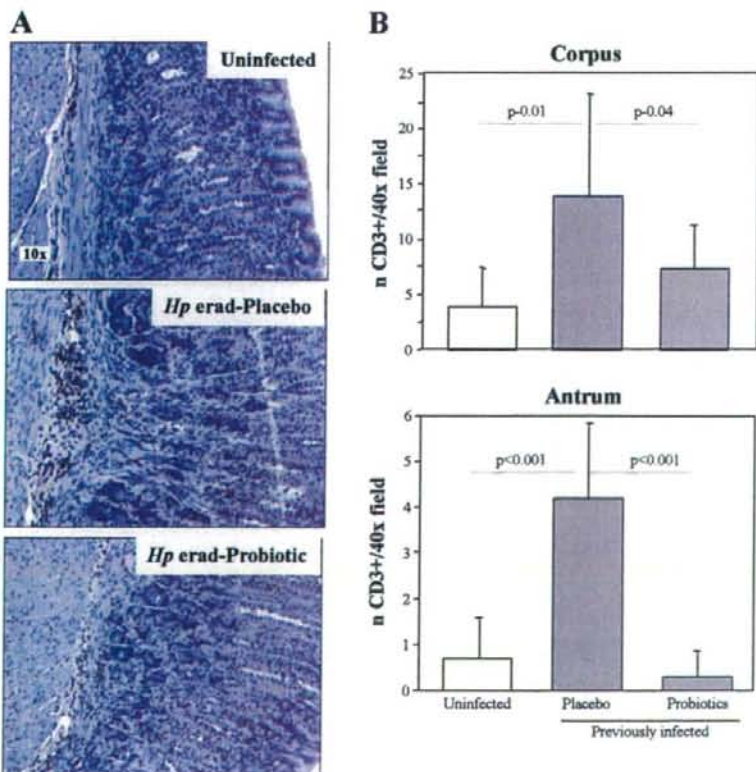


Fig. 3. A: at 2 mo posteradication, a persistent, predominantly submucosal chronic infiltrate was observed in the gastric body of mice previously infected with *H. pylori* compared with uninfected controls. Treatment with probiotics decreased chronic gastritis (photograph magnification $\times 10$). B: CD3⁺ cell counts were increased both in corpus ($P = 0.01$) and antrum ($P < 0.0001$) of previously infected mice compared with uninfected controls. Treatment with probiotics normalized the CD3⁺ cell counts in both corpus and antrum ($P = 0.04$ and $P < 0.001$ vs. placebo-treated mice). Quantification performed in mucosa and submucosa; 2 slides/mouse ($n = 12/\text{group}$) (magnification $\times 63$) and averaged.

gastric emptying values. However, there were no statistical differences between previously infected mice treated with placebo or probiotics 2 wk after eradication. Two months after eradication, previously infected mice that had been treated with probiotics tended to have faster gastric emptying than placebo-treated mice (Fig. 1).

Probiotics improve recovery of 24-h feeding behavior after *H. pylori* eradication. At 4 mo, frequency of eating bouts per 24 h was higher in *H. pylori*-infected mice compared with uninfected controls (Fig. 4). In placebo-treated mice, altered feeding patterns remained unchanged for at least 2 mo post-eradication. In contrast, previously infected mice treated with probiotics had a similar number of eating bouts per 24 h as uninfected time-point controls.

Effect of probiotics on paracellular small intestinal permeability. Only intestinal segments with intact morphology at the end of experiments were included in the study. Jejunal permeability to $^{51}\text{Cr-EDTA}$ and $^{14}\text{C-mannitol}$ was increased in *H. pylori*-infected mice compared with uninfected controls (Fig. 5). Bacterial eradication combined with probiotics tended to improve paracellular permeability to $^{51}\text{Cr-EDTA}$ ($P = 0.35$ vs. uninfected controls), but this did not reach statistical significance vs. placebo-treated mice. Probiotics did not affect membrane permeability as assessed by $^{14}\text{C-mannitol}$.

DISCUSSION

The aim of our study was to investigate whether the antigenic or bacterial content of the gut influences the rate of recovery of host physiology induced by chronic *H. pylori* infection after bacterial eradication.

We have previously shown that altered gastric emptying improves at 2 wk post-eradication and completely normalizes 2 mo post-eradication (3). The probiotic combination *L. rhamnosus* R0011 and *L. helveticus* R0052 administered immediately after *H. pylori* eradication accelerated recovery of gastric chronic inflammation. In contrast, previously infected mice that received *H. pylori* antigen had persistent CD3^+ cell counts in the stomach that correlated with persistent delayed gastric emptying post-eradication.

In the chronic model of *H. pylori* infection, the degree of neural impairment is proportional to the extent of the chronic inflammatory infiltrate (2). In the present study, we have extended this observation and showed that the degree of delayed gastric emptying is proportional to the CD3^+ cell counts in the stomach. Furthermore, *H. pylori* antigen-treated mice had persistent delayed gastric emptying post-eradication compared with placebo-treated controls. This was accompanied by higher anti-*H. pylori* antibody titers, suggesting a

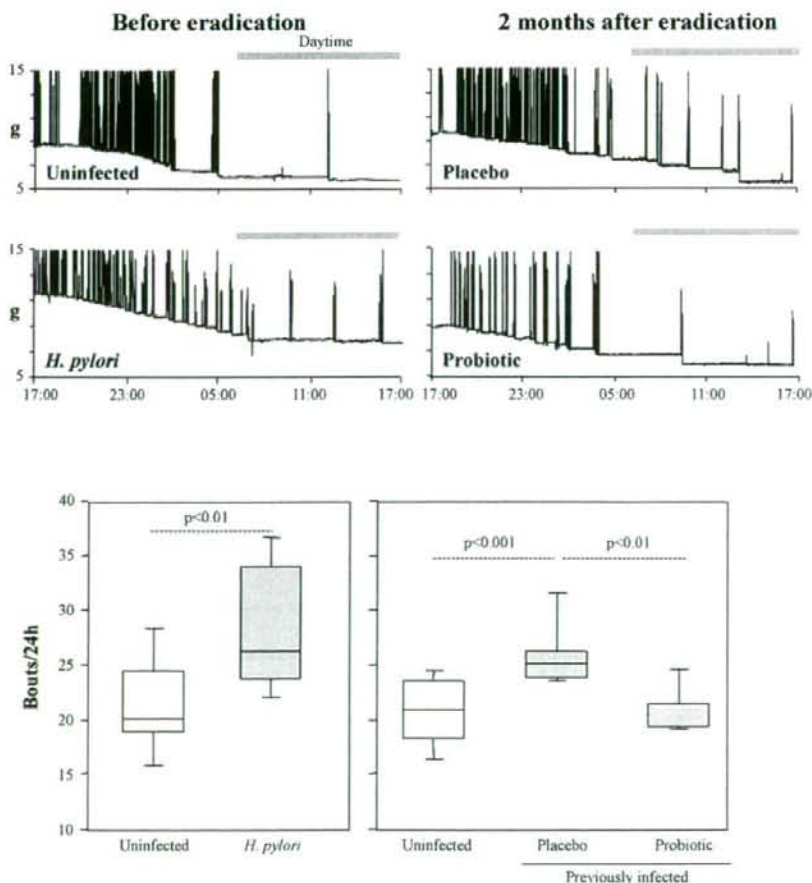


Fig. 4. Examples of feeding patterns in control ($n = 8$) and *H. pylori*-infected mice ($n = 16$). Control mice fed almost exclusively during nighttime. *H. pylori*-infected mice fed frequently during the day time as well. The abnormal feeding pattern persisted until 2 mo post-eradication in placebo-treated mice ($n = 8$), whereas it normalized in probiotic-treated mice ($n = 8$). The number of feeding bouts was increased in *H. pylori*-infected mice compared with controls. Two months post-eradication, the number of feeding bouts normalized in probiotic-treated mice but not in placebo-treated mice.

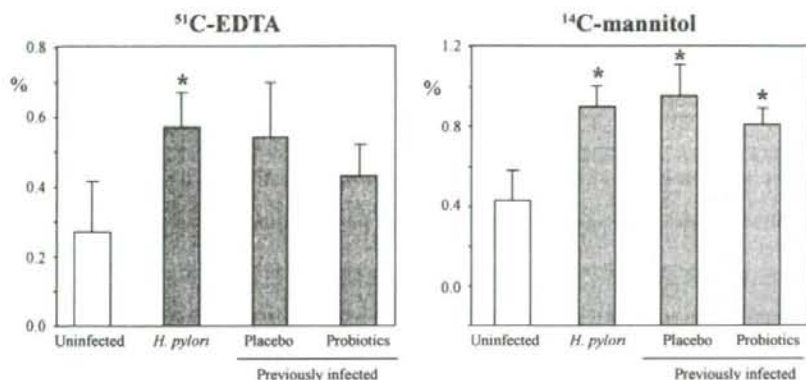


Fig. 5. Intestinal permeability for both $^{51}\text{C-EDTA}$ and $^{14}\text{C-mannitol}$ was increased in *H. pylori*-infected mice ($n = 16$) compared with uninfected controls ($n = 7$). Intestinal permeability tended to improve 2 mo posteradication in probiotic-treated mice ($n = 8$) but did not reach statistical significance vs. placebo-treated mice ($n = 8$). * $P < 0.05$ vs. uninfected controls.

heightened immune response after luminal *H. pylori* antigen administration. In contrast, probiotic therapy significantly decreased the number of CD3^+ cells in the stomach of previously infected mice compared with placebo-treated mice in parallel with a faster recovery of gastric emptying. Thus the effect of probiotics on gastric emptying recovery may be mediated through a faster recovery of the chronic inflammatory response to *H. pylori*.

H. pylori-infected mice ate more frequently but smaller amounts of food per feeding bout compared with uninfected controls. This resulted in a similar total amount of food consumed per 24 h. The pattern is reminiscent of that observed frequently in patients with functional dyspepsia who have difficulty consuming regular size meals and therefore snack frequently throughout the day. Administration of probiotics normalized postinfective altered feeding behavior. It is possible that chronic inflammation in the stomach alters ascending neural pathways, resulting in abnormal feeding behavior, and that probiotics improve this through an effect on *H. pylori*-associated gastritis. However, other mechanisms such as direct modulation of neuroendocrine pathways by probiotics cannot be ruled out.

It has been shown that *H. pylori* infection alters gastric permeability in vivo and also on epithelial cell lines (6, 14, 15). The underlying mechanisms may include impaired mucus-bicarbonate barrier, disruption of tight junctions (occludin, ZO), and increase in transcellular permeability by *H. pylori*. These alterations may be long lasting and linked to chronic inflammation because bacterial eradication has been shown to improve gastric permeability only in those mice with significant improvement of chronic gastritis (14). A recent clinical study has suggested that intestinal permeability is altered in subjects with *H. pylori* infection (5). This may be more clinically relevant than changes in gastric permeability because the intestine represents a larger area of antigen and nutrient processing. The *H. pylori*-induced defect in intestinal barrier could result in chronic immune stimulation and bystander antigen stimulation even after *H. pylori* eradication. We measured permeability in ex vivo jejunal segments using a combination of two macromolecules to assess paracellular and membrane permeability. $^{51}\text{Cr-EDTA}$ is an established marker for paracellular permeability, and C^{14} -mannitol is considered to be a marker for membrane permeability in vivo studies. Both permeabil-

ity to $^{51}\text{Cr-EDTA}$ and mannitol were increased in *H. pylori*-infected mice. Bacterial eradication did not normalize intestinal permeability. Treatment with probiotics tended to improve paracellular permeability, but this did not achieve statistical significance compared with those previously infected and treated with placebo. Probiotics did not modify membrane permeability. In contrast to the probiotic-induced normalization of feeding behavior, combined administration of *L. rhamnosus* R0011 and *L. helveticus* R0052 had a modest effect on increased intestinal permeability after bacterial eradication. Thus resolution of altered feeding behavior and gastric emptying abnormalities by probiotics do not seem to be mediated principally by an improvement in small intestinal permeability in the chronic model of *H. pylori* infection. This finding is in disagreement with an earlier study using a stress model in the rat that showed that this combination of probiotic bacteria could protect against bacterial translocation (24). However, it supports the findings in an acute model of *H. pylori* in which *L. rhamnosus* R0011 and *L. helveticus* R0052 reduced *H. pylori*-induced antrum inflammation but not apoptosis (10).

In conclusion, using a murine model of chronic *H. pylori* infection and postinfective gut dysfunction we have shown that administration of *L. rhamnosus* R0011 and *L. helveticus* R0052 after *H. pylori* eradication accelerates recovery of gastric motor function and normalizes altered feeding behavior. This is associated with improvement in chronic gastric inflammation by probiotics but not with full recovery of intestinal barrier abnormalities. Treatment with luminal antigen related to the triggering infectious agent maintains gastric dysfunction long after bacterial eradication. The results suggest that specific probiotics may be useful in improving the rate of symptomatic relief in patients with dyspepsia after *H. pylori* eradication.

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REFERENCES

- Bercik P, Armstrong D, Emde C, Dutoit P, Fraser R, Blum AL, Kucera P. Origin of rhythmic motility patterns in arterially perfused rat intestine. *Gastroenterology* 106: 649–657, 1994.
- Bercik P, De Giorgio R, Blennerhasset P, Verdú EF, Barbara G, Collins SM. Immune-mediated neural dysfunction in a murine model of chronic *Helicobacter pylori* infection. *Gastroenterology* 123: 1205–1215, 2002.
- Bercik P, Wang L, Kean I, Verdú EF, Tougas G, Collins SM. Persistence of sensory and eating disturbances after *H. pylori* eradication in mice. *Gastroenterology* 122: W1074, 2002. (Abstract)
- Blum AL, Talley NJ, O'Morrain C, van Zanten SV, Labenz J, Stolte M, Louw JA, Stubberod A, Theodors A, Sundin M, Bolling-Sternevald E, Junghard O. Lack of effect of treating *Helicobacter pylori* infection in patients with non-ulcer dyspepsia. Omeprazole plus clarithromycin and amoxicillin effect one year after treatment (OCA) study group. *N Engl J Med* 339: 1875–1881, 1998.
- Di Leo V, D'Inca R, Bettini MB, Podsiadek M, Punzi L, Mastropalo G, Sturniolo GC. Effect of *Helicobacter pylori* and eradication therapy on gastrointestinal permeability. Implications for patients with seronegative spondyloarthritis. *J Rheumatol* 32: 295–300, 2005.
- Fedwick JP, Lapointe TK, Meddings JB, Sherman PM, Buret AG. *Helicobacter pylori* activates myosin light-chain kinase to disrupt claudin-4 and claudin-5 and increase epithelial permeability. *Infect Immun* 73: 7844–52, 2005.
- Graham DY, Klein PD. *Campylobacter pyloridis* gastritis: the past, the present and speculations about the future (Review). *Am J Gastroenterol* 82: 283–286, 1987.
- Gwee KA, Graham JC, McKendrick MW, Collins SM, Marshall JS, Walters SJ, Read NW. Psychometric scores and persistence of irritable bowel after infectious diarrhoea. *Lancet* 347: 150–153, 1996.
- Jiang B, Jordana M, Xing Z, Smail F, Snider DP, Borojevic R, Steele-Norwood D, Hunt RH, Croitoru K. Replication-defective adenovirus infection reduces *Helicobacter felis* colonization in the mouse in a gamma interferon- and interleukin-12-dependent manner. *Infect Immun* 67: 4539–4544, 1999.
- Johnson-Henry KC, Mitchell DJ, Avitzur Y, Galindo-Mata E, Jones NL, Sherman PM. Probiotics reduce bacterial colonization and gastric inflammation in *H. pylori*-infected mice. *Dig Dis Sci* 49: 1095–1102, 2004.
- Malferrtheiner P, Fischbach W, Layer P, Moessner J, Stolte M, Ledolter A, Demleitner K, Fuchs WA. ELAN study proves symptomatic benefit of *Helicobacter pylori* eradication in functional dyspepsia. *Gastroenterology* 118: A440, 2000. (Abstract)
- Marshall JK, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM, Walkerton Health Study Investigators. Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. *Gastroenterology* 131: 445–450, 2006.
- Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 16: 1311–1315, 1984.
- Matysiak-Budnik T, Hashimoto K, Heyman M, de Mascarel A, Desjeux JF, Mégraud F. Antral gastric permeability to antigens in mice is altered by infection with *Helicobacter felis*. *Eur J Gastroenterol Hepatol* 11: 1371–1377, 1999.
- Matysiak-Budnik T, Terpend K, Alain S, Sanson le Pors MJ, Desjeux JF, Mégraud F, Heyman M. *Helicobacter pylori* alters exogenous antigen absorption and processing in a digestive tract epithelial cell line model. *Infect Immun* 66: 5785–5791, 1998.
- McColl K, Murray L, El-Omar E, Dickson A, El-Nujumi A, Wirz A, Kelman A, Penny C, Knill-Jones R, Hilditch T. Symptomatic benefit from eradicating *Helicobacter pylori* infection in patients with non-ulcer dyspepsia. *N Engl J Med* 339: 1869–1874, 1998.
- Mearin F, Pérez-Oliveras M, Perelló A, Vinyet J, Ibañez A, Coderch J, Perona M. Dyspepsia and irritable bowel syndrome after a *Salmonella* gastroenteritis outbreak: one-year follow-up cohort study. *Gastroenterology* 129: 98–104, 2005.
- Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. Hemoglobin vesicles as artificial oxygen carriers: present situation and future visions. *J Intern Med* 263: 4–15, 2008.
- Spiller RC, Jenkins D, Thornley JP, Hebdon JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute *Campylobacter* enteritis and in post-dysenteric irritable bowel syndrome. *Gut* 47: 804–811, 2000.
- Tack J, Demedts I, Dehondt G, Caenepeel P, Fischler B, Zandeck M, Janssens J. Clinical and pathophysiological characteristics of acute-onset functional dyspepsia. *Gastroenterology* 122: 1738–1747, 2002.
- Talley NJ, Janssens J, Lauritsen K, Racz I, Bolling-Sternevald E. Eradication of *Helicobacter pylori* in functional dyspepsia: randomised double blind placebo controlled trial with 12 mo' follow up. The optimal regimen cures *Helicobacter* induced dyspepsia (ORCHID) study group. *BMJ* 318: 833–837, 1999.
- Talley NJ, Vakil N, Ballard ED 2nd, Fenerty MB. Absence of benefit of eradicating *Helicobacter pylori* in patients with non-ulcer dyspepsia. *N Engl J Med* 341: 1106–1111, 1999.
- Varghese AK, Verdú EF, Bercik P, Khan WI, Blennerhasset PA, Szechtman H, Collins SM. Antidepressants attenuate increased susceptibility to colitis in a murine model of depression. *Gastroenterology* 130: 1743–1753, 2006.
- Wang LH, Fang XC, Pan GZ. Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis. *Gut* 53: 1096–1101, 2004.
- Zareie M, Johnson-Henry K, Jury J, Yang PC, Ngan BY, McKay DM, Soderholm JD, Perdue MH, Sherman PM. Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. *Gut* 55: 1553–1560, 2006.



Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: Effect of the structure of acidic groups

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Abstract

Anionic vesicles containing acidic phospholipids are known complement activators. To clarify which negative physicochemical electrostatic charges on vesicles and structural specificities of acidic lipids are critical to complement activation, the electrostatic properties and activity to complement of two anionic vesicles modified with a carboxylic acid derivative or a conventional acidic phospholipid were compared. Electrophoretic mobility measurements indicated that the negative zeta potential and the electrostatic interactivity of these two anionic vesicles were equal at pH 7.4. However, the infusion of vesicles containing acidic phospholipid induced significant complement activation, while vesicles containing the carboxylic acid derivative failed to activate complement. These results indicate that the negative charge on the surface of vesicles is not critical for the activation complement, suggesting that complement activation is specific to the structure of acidic groups. This finding is likely to be important to the design of anionic biointerfaces and may support the promising medical applications of this anionic vesicle modified with a carboxylic acid derivative.

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Keywords: Liposome; Acidic lipid; Anionic surface; Electrostatic interaction; Complement activation

1. Introduction

Anionic vesicles (liposomes), commonly formulated by mixing acidic phospholipids such as phosphoglycerol, phosphoserin, phosphoinositol, phosphatidic acid, cardiolipin, and poly (ethylene glycol) (PEG) conjugated phosphoethanolamine in lipid components are known to mediate complement activation [1–5]. Complement activation, which is followed by systemic immune activation and anaphylaxis shock, is regarded as a critical problem in the clinical setting of various biomaterials. The C1q subcomponent of C1 has a highly cationic region in residues 14–26 of the C1qA polypeptide chain, and this specific region of the collagenous stalk of C1q has been identified as being involved in interactions with negatively charged activators [5–8]. It is

believed that the antibody-independent binding of C1q to the negatively charged surface of vesicles initiates the activation of the complement cascade via the classical complement pathway [2]. To prevent complement activation, the acidic phospholipids can be removed or their negative charge can be protected by chemical modification [2,4]. It has also been reported that surface modification with a dense PEG layer is effective for preventing complement activation by covering the surface charge [9].

Our group has developed phospholipid vesicles called hemoglobin-vesicles that encapsulate human hemoglobin and that can be used as a substitute for red blood cells and as an alternative to conventional transfusion [10–14]. To achieve this challenging application of vesicles, we had to develop anionic vesicles capable of encapsulating hemoglobin using a minimum amount of lipids, retaining the negative charge on the membrane which reduces the lamellarity of vesicles and is required for improving encapsulation capacity [11–13]. Several acidic lipids have been tested and a carboxylic acid derivative, L-glutamic

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acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA), has been used as an anionic component. Vesicles containing SA have been demonstrated to efficiently encapsulated hemoglobin, and given their stability, can be infused in considerably large doses as a red blood cell substitute [15,16]. In addition, administration of this formulation is not associated with considerable rejection by the blood immune system and complement in animals [17,18]. Recent collaboration involving our group found that vesicles containing SA are selectively captured by bone marrow macrophages at small injection doses in rabbits [19]. This targeting of bone marrow has not yet been reported for conventional anionic vesicles. These findings suggest that the surface characteristics of the anionic vesicles have a marked effect on their associated biological events and that this is highly dependent upon the nature of the acidic groups.

Based on the hypothesis that the negative electrostatic charge of acidic phospholipids on the surface of vesicles is one of the factors responsible for inducing complement activation, the electrostatics of vesicles containing SA may differ from those of vesicles containing acidic phospholipids. Conversely, when the electrostatic interactivity of these acidic lipids is equal, biological events such as complement activation are likely to be mediated by the molecular specificity of acidic lipids. To elucidate what aspects are critical to the biocompatibility and performance of anionic vesicles, a comparative analysis of the electrostatic interfacial properties of vesicles containing either SA or acidic phospholipids is essential. In this study, we elucidated the electrostatic interfacial properties of vesicles containing SA or an acidic phospholipid by electrophoretic mobility measurement and observed complement activation after the infusion of these vesicles in animals. The results clearly demonstrated that the negatively charged group on the surface of anionic vesicles is not critical to the activation of complement.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (PC), cholesterol (CH), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (PG), and L-glutamic acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA), were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)] (PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). Pentyllysine and poly-L-lysine (Mw. 15–30 kDa) were purchased from SIGMA (St. Louis, MO).

2.2. Preparation of vesicles

Vesicle samples were produced using PC and CH (1:1, molar ratio, PC-vesicles), and varying amounts of SA (SA-vesicles) or PG (PG-vesicles). The mixed lipids were added to 10 mM phosphate buffer (NaCl; 20 mM, pH 7.4) and the dispersion was introduced into an extruder (Lipex Biomembrane, Canada) and extruded through the membrane filters (final pore size: 0.2 μ m, Isopore[®], Millipore, Tokyo, Japan) under pressure using nitrogen gas. For animal experiments, vesicles were composed of PC, CH, and SA or PG (1:1:0.2, molar ratio), with 0.3 mol% of PEG-DSPE incorporated to prevent the aggregation of vesicles [20]. Samples for animal experiments were prepared under sterile conditions.

2.3. Characterization of vesicles

The diameters of the resulting vesicles were determined with a COULTER submicron particle analyzer (N4SD, Coulter, Hialeah, FL), and the average

diameter \pm standard deviation (SD) was calculated. The phospholipid concentration was determined using a cholineoxidase method (Phospholipid C Test Wako; Wako Pure Chem., Tokyo). Endotoxin contamination in the samples prepared for the animal experiment was determined to be less than 0.1 EU/mL by the Limulus assay test [21].

2.4. Determination of zeta potential

A 10 μ L aliquot of vesicles (lipid concentration: 2 g/dL) was diluted in 2 mL of 10 mM phosphate buffer (pH 3–9, 37 $^{\circ}$ C) containing 20 mM NaCl and incubated for 1 h at 37 $^{\circ}$ C. The pH was determined using a pH meter with a pH electrode (F-52, HORIBA, Kyoto, Japan) at 37 $^{\circ}$ C and the electrophoretic mobility of vesicles (lipid concentration: 0.01 g/dL) were determined by Laser Doppler Velocimetry (Zeta-Sizer Nano ZS, Malvern Instruments, Malvern, Worcestershire, UK). Measurement conditions and parameters were as follows: 37 $^{\circ}$ C, dielectric constant 74.4 (for dilute water solution), viscosity 0.6864 cP (for dilute water solution), and the applied voltage was 20 V/cm. The zeta potential was determined by measuring the electrophoretic mobility (U_E), and the zeta potential (ζ) and applied to the Henry equation:

$$U_E = \frac{2\varepsilon f(\kappa R)}{3\eta} \zeta \quad (1)$$

where ε is the dielectric constant, η is the viscosity of the solvent, $f(\kappa R)$ is the Henry function, κ is the Debye–Hückel parameter and the R is the radius of the particle. The Smoluchowski equation was used with $f(\kappa R) = 1.5$ employed in the zeta potential calculations. Measurements were performed four times for each sample and statistical analysis was conducted using the average \pm SD of the four measurements.

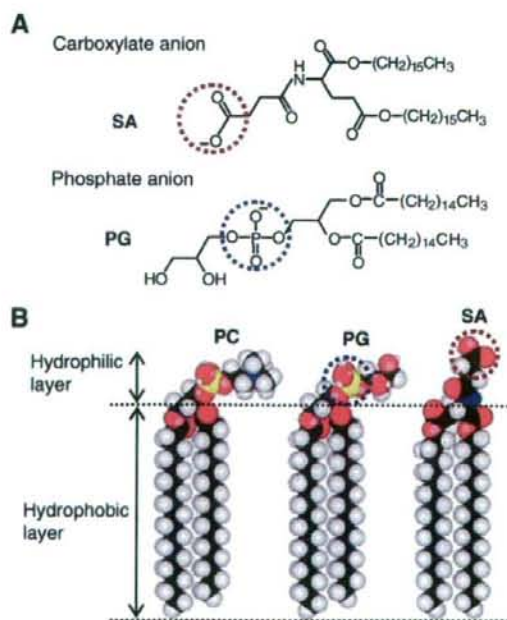


Fig. 1. Structure of acidic lipids for surface modification of vesicles. (A) Chemical structure of L-glutamic acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (PG). The focus of this study is the different acidic groups shown in the dotted circle. (B) The CPK model of SA and PG with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC) to estimate the distance of acidic groups from the membrane surface of PC. CPK models show the structure with minimized energy in molecular mechanistic calculation.