stabilized in order to prevent intervesicular aggregation, fusion and leakage of the encapsulated Hb.

The surface modification of phospholipid vesicles with poly(ethylene glycol) (PEG)-conjugated lipid is a well-known method of prolonging the circulation time of the vesicles *in vivo* for drug delivery systems (Klibanov *et al.*, 1990). For HbV, the surface was also modified with PEG chains to improve the dispersion state of the vesicles when mixed with blood components (Yoshioka, 1991). The PEG-modified HbV has shown an improved blood circulation and tissue oxygenation due to the absence of HbV aggregate formation and viscosity elevation (Sakai *et al.*, 1997, 1998) and prolonged circulation persistence *in vivo* (Sou *et al.*, 2003b). However, little attention has been paid to the ability of the PEG modification for the long-term preservation of vesicles or liposomes in the liquid state. We studied the possibility of the long-term preservation of Hb vesicles by the combination of two technologies – surface modification of HbV with PEG chains, and deoxygenation during storage for 2 years (Sakai *et al.*, 2000c). The samples stored at 4° and 23°C showed a stable dispersion state for 2 years, though the sample stored at 40°C underwent precipitation and decomposition of the vesicular components, a decrease in pH, and 4 per cent leakage of the total Hb after 1 year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion due to their steric hindrance. The original metHb content (~3 per cent) before the preservation gradually decreased to less than 1 per cent in all the samples after 1 month due to the presence of homocysteine inside the vesicles, which consumed the residual O_2 (thiol groups in homocysteines reacted with oxygen to generate disulfide and active oxygen species) and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the O_2 partial pressure, and no increase in the metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. These results clearly indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years.

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the application of PEG-modified vesicles in other fields. The long-term preservation of O_2 carriers overcomes the limitation of the blood transfusion system and will contribute to benefiting clinical medicine.

ENDOTOXIN

The production process of HbV has to be guaranteed by a good manufacturing practice (GMP) standard as a biological product regarding the strict regulation of impurities and viral and bacterial contamination. Monitoring the content of the lipopolysaccharide (LPS), known as an endotoxin, a component of the outer membrane of Gram-negative bacteria possessing a large variety of biological influences on numerous mammalian cells and tissues, is strictly required. The US FDA has established a guideline for the human maximal endotoxin dose permissible for parenteral products (5 EU/kg) that may include HBOCs. This limit is based on the endotoxin activity (Endotoxin Unit: EU; 1 EU = 100 pg), and can be measured via the *Limulus amebocyte* lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS (Levin and Bang, 1964). Since the volume of O_2 carriers to be infused for shock resuscitation or acute hemodilution is estimated to be less than 20 ml/kg, the specific endotoxin limits per ml should be 0.25 EU/ml (= 15/20), similar to that for water for injection (0.25 EU/ml).

Bacterial LPS is a gigantic amphiphilic macromolecule, therefore it interacts hydrophobically with protein and biomembranes. Hb strongly interacts with LPS, showing synergistic toxicity. The constituent of endotoxin that causes LAL gelation is a glycophospholipid – designated lipid-A. Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A, such as its LAL gelation activity (Richardson *et al.*, 1983). As a consequence, the researchers who have studied HbV or other phospholipid vesicles for delivering other functional molecules have encountered a problem in measuring the LPS content for the quality control of these materials (Cliff *et al.*, 1995; Harmon *et al.*, 1997).

Considering this background information, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) to release the LPS entrapped in the vesicles as a pretreatment for the subsequent LAL assay of the kinetic-turbidimetric gel clotting analysis using a Toxinometer® (Sakai *et al.*, 2004e). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition

that allowed the measurement of LPS higher than 0.1 EU/ml in the HbV suspension.

This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer® is routinely used in our HbV production system. Significant attention is paid to the quality control of HbV for preclinical studies, and all the HbV prepared under sterile conditions showed an LPS content less than 0.2 EU/ml at [Hb] = 10 g/dl. Moreover, the utilization of the histidine-immobilized agarose gel (Pyrosep®) effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/ml. The pretreatment with $C_{12}E_{10}$ would be applicable not only to HbV but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules.

HEMOGLOBIN VESICLES AS OXYGEN CARRIERS *IN VIVO*

The advantages of HbV and other HBOCs are the absence of blood-type antigens and infectious viruses, and stability for long-term storage that outdoes the RBC transfusion. The shorter half-lives of the HBOCs in the bloodstream (2–3 days) limit their use, but they are applicable for a shorter period of use, such as (1) a resuscitative fluid for hemorrhagic shock during an emergency situation for a temporary time or bridging until the packed RBCs are available; (2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion; (3) a priming solution for the circuit of an extacorporeal membrane oxygenator (ECMO) (Yamazaki *et al.*, 2004); and (4) other potential indications, e.g. so-called O_2 therapeutics to oxygenate ischemic tissues.

One particle of HbV (diameter ~250 nm) contains about 30 000 Hb molecules. Since HbV acts as a particle in the blood and not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates for plasma expanders are HSA, hydroxyethyl starch, dextran or gelatin, depending on the clinical setting, cost, country concerned and clinicians. Recombinant human serum albumin (rHSA) is the alternative. The absence of any infectious disease from humans is the greatest advantage of rHSA, which will be soon approved for clinical use in Japan. Moreover, there should be no immunological and hematological abnormalities, which are often seen when using dextran and hydroxyethyl starch. Aimed at the application of HbV suspended in a plasma expander for the above indications, HbV was tested in resuscitation from hemorrhagic shock (Sakai *et al.*, 2002b, 2004a; Yoshizu *et al.*, 2004) and extreme hemodilution (Izumi *et al.*, 1997; Kobayashi *et al.*, 1997; Sakai *et al.*, 1997, 1998, 1999) in collaboration with Waseda–Keio and Professor Marcos Intaglietta at UCSD. Moreover, HbV with a high O_2 affinity (low *P*50) suspended in HES or dextran was tested for oxygenation of an ischemic skin flap by Erni *et al.* at the Inselspital University Hospital, Berne (Contaldo *et al.*, 2003; Erni *et al.*, 2003) and the results imply the further application of HbV for other ischemic diseases such as myocardial and brain infarction, and stroke.

SUMMARY

Based on the above establishment of the HbV production system and the potential clinical applications of HbV, significant efforts have been made in the joint collaboration partnership of Waseda–Keio–Oxygenix–Nipro to produce HbV with a facility of GMP standard, and to start preclinical and, finally, clinical trials. Since the combination of recombinant Hb (rHb)-vesicles suspended in recombinant albumin (rHSA) would be the ideal 'artificial red blood cells', this project has recently initiated the next generation HbV (Kai *et al.*, 2004).

EDITOR'S SUMMARY

Liposome encapsulated hemoglobin is a long-sought goal in Japan, where the product is called hemoglobin vesicles (HbV), which distinguishes this product from the one developed primarily in the US, whose designation is LEH. HbV is the result of a long series of studies in which the size of the vesicles, including the number of lipid layers, the surface composition and materials co-encapsulated have been optimized.

HbV is produced by an extrusion process that has commercial potential, although at this time the product has not yet been produced in

quantities sufficient for clinical trials. Sterilization of the hemoglobin, prior to encapsulation, is performed using heat, and antioxidants are co-encapsulated to retard hemoglobin oxidation. Oxygen affinity is regulated to any desired *P*50 by co-encapsulation of allosteric effectors, and this group has contributed important studies on the effect of different *P*50 on oxygen delivery to tissues by HbV. The product is claimed to be stable when stored for up to 2 years.

A commercial effort has been launched in Japan, and it is hoped that HbV could be in human clinical trials within the next few years.

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REFERENCES

Abe, H., Ikebuchi, K., Hirayama, J. *et al.* (2001). Virus inactivation in hemoglobin solution by heat treatment. *Artif. Cells Blood Subst. Immob. Biotech.*, **29**, 381–388.

Bangham, A. D. and Horne, R. W. (1964). Negative staining of phospholipids and their structure modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.*, **8**, 660–668.

Chang, T. M. S. (1991). Blood substitutes based on modified hemoglobin prepared by encapsulation or crosslinking: an overview. *Biomat. Artif. Cells Immob. Biotech.*, **20**, 159–182.

Chung, J. E., Hamada, K., Sakai, H. *et al.* (1995). Ligand exchange reaction of carbonylhemoglobin to oxyhemoglobin in a hemoglobin liquid membrane. *Nippon Kagaku Kaishi*, **1995**, 123–127.

Cliff, R.O., Kwasiborski, V. and Rudolph, A.S. (1995). A comparative study of the accurate measurement of endotoxin in liposome encapsulated hemoglobin. *Artif. Cells Blood Subst. Immob. Biotech.*, **23**, 331–336.

Contaldo, C., Schramm, S., Wettstein, R. *et al.* (2003). Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol.*, **285**, H1140–H1147.

Djordjevich, L. and Miller, I. F. (1977). Lipid encapsulated hemoglobin as a synthetic erythrocyte. *Fed. Proc.*, **36**, 567.

Erni, D., Wettstein, R., Schramm, S. *et al.* (2003). Normovolemic hemodilution with hemoglobin vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am. J. Physiol. Heart Circ. Physiol.*, **284**, H1702–H1709.

Fukutomi, I., Sakai, H., Takeoka, S. *et al.* (2002). Carbonylation of oxyhemoglobin solution using a membrane oxygenator. *J. Artif. Organs*, **5**, 102–107.

Goda, N., Suzuki, K., Naito, M. *et al.* (1998). Distribution of heme oxygenase isoforms in rat liver.Topographic basis for carbon monoxide-mediated microvascular relaxation. *J. Clin. Invest.*, **101**, 604–612.

Harmon, P., Cabral-Lilly, D., Reed, R. A. *et al.* (1997). The release and detection of endotoxin from liposomes. *Anal. Biochem.*, **250**, 139–146.

Huang, Y., Takeoka, S., Sakai, H. *et al.* (2002). Complete deoxygenation from a hemoglobin solution by an electrochemical method and heat treatment for virus inactivation. *Biotechnol. Progr.*, **18**, 101–107.

Ito, T., Fujihara, M., Abe, H. *et al.* (2001). Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on N-formyl-methionyl-leucyl-phenylalanine-induced responses of polymorphonuclear neutrophils *in vitro*. *Artif. Cells Blood Subst. Immob. Biotech.*, **29**, 427–437.

Izumi, Y., Sakai, H., Hamada, K. *et al.* (1996). Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical oxygen tension. *Crit. Care Med.*, **24**, 1869–1873.

Izumi, Y., Sakai, H., Takeoka, S. *et al.* (1997). Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *Am. Soc. Artif. Int. Organs J.*, **43**, 289–297.

Kai, T., Kida, Y., Fukutomi, I. *et al.* (2004). Development of totally synthetic oxygen carriers. *Artif. Blood*, **13**, 34–41.

Kerwin, B. A., Akers, M. J., Apostol, I. *et al.* (1999). Acute and long-term stability studies of deoxy hemoglobin and characterization of ascorbate-induced modifications. *J. Pharm. Sci.*, **88**, 79–88.

Klibanov, A. L., Maruyama, K., Torchilin, V. P. and Huang, L. (1990). Amphipathic polyethylene glycol effectively prolongs the circulation time of liposomes. *FEBS Lett.*, **268**, 235–237.

Kobayashi, K., Izumi, Y., Yoshizu, A. *et al.* (1997). The oxygen carrying capability of hemoglobin vesicles evaluated in rat exchange transfusion models. *Artif. Cells Blood Subst. Immob. Biotech.*, **25**, 357–366.

Levin, J. and Bang, F. B. (1964). The role of endotoxin in the extracellular coagulation of limulus blood. *Bull. Johns Hopkins Hosp.*, **115**, 265–274.

Levy, A., Zhang, L. and Rifkind, J. M. (1988). Hemoglobin: a source of superoxide radical under hypoxic conditions. *Oxy-radicals Mol. Pathol. Proc. Upjohn-UCLA Symp.*, 11–25.

Murray, J. A., Ledlow, A., Launspach, J. *et al.* (1995). The effects of recombinant human hemoglobin on esophageal motor function in humans. *Gastroenterology*, **109**, 1241–1248.

Naito, Y., Fukutomi, I., Masada, Y. *et al.* (2002). Virus removal from hemoglobin solution using Planova membrane. *J. Artif. Organs*, **5**, 141–145.

Ogata, Y., Goto, H., Kimura, T. and Fukui, H. (1997). Development of neo red cells (NRC) with the enzymatic reduction system of methemoglobin. *Artif. Cells Blood Subst. Immob. Biotech.*, **25**, 417–427.

Page, T. C., Light, W. R., McKay, C. B. and Hellums, J. D. (1998). Oxygen transport by erythrocyte/hemoglobin solution mixtures in an *in vitro* capillary as a model of hemoglobin-based oxygen carrier performance. *Microvasc. Res.*, **55**, 54–66.

Richardson, E. C., Banerji, B., Seid, R. C. Jr *et al.* (1983). Interactions of lipid A and liposome-associated lipid A with *Limulus polyphemus* amoebocytes. *Infect. Immun.*, **39**, 1385–1391.

Rudolph, A. S., Klipper, R. W., Goins, B. and Phillips, W. T. (1991). *In vivo* biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. *Proc. Natl. Acad. Sci. USA*, **88**, 10976–10980.

Sakai, H., Takeoka, S., Yokohama, H. *et al.* (1993). Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expr. Purif.*, **4**, 563–569.

Sakai, H., Hamada, K., Takeoka, S. *et al.* (1996). Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol. Progr.*, **12**, 119–125.

Sakai, H., Takeoka, S., Park, S. I. *et al.* (1997). Surface-modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90 per cent-exchange transfusion in anesthetized rats. *Bioconjug. Chem.*, **8**, 23–30.

Sakai, H., Tsai, A. G., Kerger, H. *et al.* (1998). Subcutaneous microvascular responses to hemodilution with red cell substitutes consisting of polyethylene glycol-modified vesicles encapsulating hemoglobin. *J. Biomed. Mater. Res.*, **40**, 66–78.

Sakai, H., Tsai, A. G., Rohlfs, R. J. *et al.* (1999). Microvascular responses to hemodilution with Hb vesicles as red cell substitutes: influences of O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol.*, **276**, H553–H562.

Sakai, H., Hara, H., Yuasa, M. *et al.* (2000a). Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol.*, **279**, H908–H915.

Sakai, H., Yuasa, M., Onuma, H. *et al.* (2000b). Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjug. Chem.*, **11**, 56–64.

Sakai, H., Tomiyama, K., Sou, K. *et al.* (2000c). Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin vesicles as oxygen carriers in a liquid state. *Bioconjug. Chem.*, **11**, 425–432.

Sakai, H., Onuma, H., Umeyama, M. *et al.* (2000d). Photoreduction of methemoglobin by irradiation in near-ultraviolet region. *Biochemistry*, **39**, 14595–14602.

Sakai, H., Horinouchi, H., Tomiyama, K. *et al.* (2001). Hemoglobin vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am. J. Pathol.*, **159**, 1079–1088.

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

Sakai, H., Takeoka, S., Wettstein, R. *et al.* (2002b). Systemic and microvascular responses to the hemorrhagic shock and resuscitation with Hb vesicles. *Am. J. Physiol. Heart Circ. Physiol.*, **283**, H1191–H1199.

Sakai, H., Tomiyama, K., Masada, Y. *et al.* (2003a). Pretreatment of serum containing Hb vesicles (oxygen carriers) to avoid their interference in laboratory tests. *Clin. Chem. Lab. Med.*, **41**, 222–231.

Sakai, H., Suzuki, Y., Kinoshita, M. *et al.* (2003b). O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs. *Am. J. Physiol. Heart Circ. Physiol.*, **285**, H2543–H2551.

Sakai, H., Horinouchi, H., Masada, Y. *et al.* (2004a). Hemoglobin vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit. Care Med.*, **32**, 539–545.

Sakai, H., Horinouchi, H., Masada, Y. *et al.* (2004b). Metabolism of hemoglobin vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials*, **25**, 4317–4325.

Sakai, H., Masada, Y., Horinouchi, H. *et al.* (2004c). Physiologic capacity of reticuloendothelial system for degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J. Pharmacol. Exper. Ther.*, **311**, 874–884.

Sakai, H., Masada, Y., Onuma, H. *et al.* (2004d). Reduction of methemoglobin via electron transfer from photoreduced flavin: restoration of O_2-binding of

concentrated hemoglobin solution co-encapsulated in phospholipid vesicles. *Bioconjug. Chem.*, **15**, 1037–1045.

Sakai, H., Hisamoto, S., Fukutomi, I. *et al.* (2004e). Detection of lipopolysaccharide in hemoglobin vesicles by *Limulus amebocyte* lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. *J. Pharm. Sci.*, **93**, 310–321.

Shirasawa, T., Izumizaki, M., Suzuki, Y. *et al.* (2003). Oxygen affinity of hemoglobin regulates O_2 consumption, metabolism, and physical activity. *J. Biol. Chem.*, **278**, 5035–5043.

Sou, K., Naito, Y., Endo, T. *et al.* (2003a). Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol. Progr.*, **19**, 1547–1552.

Sou, K., Klipper, R., Goins, B. *et al.* (2005). Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitue. *J. Pharmacol. Exp. Ther.*, **312**, 702–709.

Takeoka, S., Sakai, H., Nishide, H. and Tsuchida, E. (1993). Preparation conditions of human hemoglobin vesicles covered with lipid membranes. *Jpn J. Artif. Organs*, **22**, 566–569.

Takeoka, S., Terase, K., Sakai, H. *et al.* (1994a). Interaction between phosphoslipid assemblies and hemoglobin (Hb). *J. Macromol. Sci. Pure Appl. Chem.*, **A31**, 97–108.

Takeoka, S., Sakai, H., Terease, K. *et al.* (1994b). Characteristics of Hb vesicles and encapsulation procedure. *Artif. Cells Blood Subst. Immob. Biotech.*, **22**, 861–866.

Takeoka, S., Ohgushi, T., Terase, K. and Tsuchida, E. (1996). Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir*, **12**, 1755–1759.

Takeoka, S., Sakai, H., Kose, T. *et al.* (1997). Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. *Bioconjug. Chem.*, **8**, 539–544.

Takeoka, S., Teramura, Y., Atoji, T. and Tsuchida, E. (2002). Effect of Hb-encapsulation with vesicles on H_2O_2 reaction and lipid peroxidation. *Bioconjug. Chem.*, **13**, 1302–1308.

Teramura, Y., Kanazawa, H., Sakai, H. *et al.* (2003). The prolonged oxygen-carrying ability of Hb vesicles by co-encapsulation of catalase *in vivo*. *Bioconjug. Chem.*, **14**, 1171–1176.

Tsuchida, E. (ed.) (1995). *Artificial Red Cells: Materials, Performances, and Clinical Study as Blood Substitutes*. John Wiley, New York.

Tsuchida, E. (ed.) (1998). *Blood Substitutes: Present and Future Perspectives*. Elsevier, Chichester.

Vandegriff, K. D. and Olson, J. S. (1984). The kinetics of O_2 release by human red blood cells in the presence of external sodium dithionite. *J. Biol. Chem.*, **259**, 12609–12618.

Wakamoto, S., Fujihara, M., Abe, H. *et al.* (2001). Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on agonist-induced platelet aggregation and RANTES release *in vitro*. *Artif. Cells Blood Subst. Immob. Biotech.*, **29**, 191–201.

Wang, L., Morizawa, K., Tokuyama, S. *et al.* (1992). Modulation of oxygen-carrying capacity of artificial red cells (ARC). *Polymer Adv. Technol.*, **4**, 8–11.

Yamazaki, M., Aeba, R. and Yozu, R. (2004). Feasibility test of artificial red cells as a priming solution for the extracorporeal membrane oxygenator. *Artif. Blood*, **12**, 45 (abstract).

Yoshioka, H. (1991). Surface modification of haemoglobin-containing liposomes with poly(ethylene glycol) prevents liposome aggregation in blood plasma. *Biomaterials*, **12**, 861–864.

Yoshizu, A., Izumi, Y., Park, S. I. *et al.* (2004). Hemorrhagic shock resuscitation with an artificial oxygen carrier hemoglobin vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma necrosis factor alpha (TNFα). *Am. Soc. Artif. Int. Organs J.*, **50**, 458–463.

CHAPTER 160

Blood Substitutes' Efficacy
Microvascular and Rheological Determinants

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The development of a blood substitute, also called "artificial" blood, or more exactly an oxygen-carrying plasma expander (OCPE), is still a major goal of transfusion medicine, driven by blood shortages, problems associated with the transmission of disease by available blood, and the complex logistics of acquiring, analyzing, storing, distributing, and delivering the needed blood. To date blood is unequaled (or appears to be) in its capacity to restore circulatory volume; however, it is often remarked that if blood were proposed today as an oxygen-carrying volume restoration fluid, it would not be approved by regulatory agencies.

Blood is needed in the presence of blood losses; however, the initial anemia is usually inconsequential to the organism's survival, whereas the associated hypovolemia is tolerated only within a narrow margin. As a result, a blood substitute should ideally target both events, possibly sequentially. Therefore, the development of an effective blood substitute is also in part related to availability and detailed understanding of an effective plasma expander, which is the term used to describe a volume restoration fluid used prior to reaching the transfusion trigger, or the point in volume restitution at which the introduction of an oxygen carrier, blood, is determined to be essential.

Blood exerts its principal functions in the microcirculation, the locale of exchange of the materials that it transports. Thus any attempt to introduce a blood surrogate must ensure efficacy at this level. In contrast, most efforts aimed at developing artificial blood were made in the absence of detailed information and analysis of how oxygen is managed at the level of the microcirculation. The cornerstones in the development of artificial blood up to the present are that this fluid should restore most of the oxygen-carrying capacity of the shed blood, that it is beneficial for the resulting mixture of remaining blood and resuscitation fluid (e.g., OCPE) to have a viscosity lower than that of natural blood, and that the material should have very low oxygen affinity so that oxygen would be readily released when blood arrives to the microcirculation.

These principles have guided the development of products that are now undergoing clinical trials. It is apparent that the initial impetus for the development of artificial blood was based on the restoration of systemic functions after acute blood losses with little or no emphasis on understanding the behavior of the resuscitation fluids in the microcirculation, which is the organ system where blood performs its functions. This was in part due to the imperfect understanding of how oxygen is managed in the microcirculation in both normal and pathophysiological conditions, the role and regulation of shear stress–dependant mediators produced by the endothelium, and the lack of techniques for measuring the key transport parameters that determine efficacy in maintaining microvascular function upon the introduction of a blood surrogate.

Oxygen-carrying capacity and oncotic pressure were prescribed to be similar to that of blood, while the experience with hemodilution suggested that improvements in transport would be obtained by lowering blood viscosity to values significantly below those of whole blood. An additional

presumed beneficial modification was the use of oxygen carriers based on modified hemoglobins with high p50s, presumed to facilitate oxygen unloading and tissue oxygenation. As acellular modified molecular hemoglobin became the oxygen carrier of choice, it was found this material was vasoactive, causing hypertension, which is deleterious in resuscitation. Vasoactivity was attributed to hemoglobin scavenging NO, leading to vasoconstriction, which gave rise to a significant effort aimed at modifying the hemoglobin molecule so that its affinity for NO was reduced.

At present an optimal OCPE is still perceived to have the following properties: oxygen-carrying capacity equivalent to 10 to 14 g/dL hemoglobin, p50 greater than 30 mmHg, viscosity 1 cP, oncotic pressure approximately 25 mmHg, and low NO binding. However, studies in the microcirculation show that a fluid configured according these concepts yields problematic outcomes in terms of resuscitation from anemic hypovolemia. Furthermore, since the most favored source of hemoglobin is human, even if it were possible to obtain a one to one conversion from blood to blood substitute, the problem of blood shortages is not solved. In an effort to circumvent the human hemoglobin source there have been various attempts to obtain hemoglobin by recombinant technology. Biopure Inc. (Boston, MA) has progressed to Phase III clinical trials with a molecular hemoglobin-based fluid derived from bovine blood. However, recombinant technology has not progressed to the development of an efficacious product to date, and the bovine-based product was not developed on the basis of microcirculatory data.

The Design of an Efficacious Oxygen-Carrying Plasma Expander

To date there are virtually no rivals to hemoglobin as a transporter of oxygen from the lung to the tissue because of its ability to bind a large amount of oxygen through a chemical reaction. The discovery that fluorocarbons could dissolve a comparatively large amount of oxygen, albeit at high oxygen partial pressures, suggested using this vehicle as the oxygen transporter. However, the use of fluorocarbon-based blood replacement fluids has not materialized, in part because of the lack of definitive experimental studies on the physiology related to altered blood physical properties and changes in the distribution of oxygen partial pressure in the circulation.

Various modifications of hemoglobin have optimized its performance and mostly eliminated the vasoactivity of this molecular species. Human hemoglobin remains the most favored source because of the well-defined methodology for obtaining blood from donors, which in advanced medical systems is virtually free of parasitic, bacterial, or viral contamination. The present formulations of bovine hemoglobin appear to be vasoactive and in the long term could present unknown risks of introducing diseases that may have extraordinarily long incubation times.

If the perceived and now frequently reported blood shortage is the driving force behind the development of hemoglobin-based oxygen-carrying blood substitutes, then the use of human hemoglobin is problematic since the processing technology and formulation would require that a unit of original blood yield at least an equivalent unit of "artificial blood," a zero-sum result that does not relieve shortages. A realistic process should produce several units of equivalent hemoglobin-based oxygen-carrying blood substitute from a unit of natural blood.

The present goal in devising a human hemoglobin-based blood substitute is to circumvent the inherent toxicity of the hemoglobin molecule and to be as efficacious as an equivalent unit of natural blood but at a lower hemoglobin concentration than blood, thus introducing a multiplying factor between the original source of human hemoglobin and the final product. Advances in microvascular technology allow us to critically analyze each of the "cornerstone" precepts that have guided the development of blood substitutes to date, namely the viscosity of the material, the affinity for oxygen, the effective concentration, and the resulting colloidal osmotic pressure when the material is present in the circulation. In the following these parameters will be analyzed from a microcirculatory perspective.

The Role of Viscosity in Oxygen Transport

Blood viscosity depends on red blood cell concentration (hematocrit) and on plasma viscosity. The manipulation of these two viscosities is the basis of the clinical practice of hemodilution. Accordingly, the restitution of blood losses with conventional plasma expanders can be effectively and safely accomplished up to a 50 percent loss of the red blood cell mass, using fluids with plasma-like viscosity. The decrease in viscosity due to hemodilution causes a compensatory increase in cardiac output due to the lowered flow resistance, thus maintaining oxygen delivery.

A specific decrease in oxygen-carrying capacity is one of the parameters that defines the so-called transfusion trigger. Microcirculatory experimental studies do not support the contention that lack of oxygen-carrying capacity is the actual determining factor in the decision of transfusing blood. In the awake hamster window chamber model, neither oxygen-carrying capacity nor tissue oxygenation is in jeopardy with red blood cell losses of two-thirds of the original mass (Figure 1).

A factor that is significantly changed upon reaching the transfusion trigger is blood viscosity, which is approximately half of normal, because of the loss of red blood cells. Thus, additional losses of red blood cells will further reduce blood viscosity, which is strongly dependent on hematocrit. The reduction of blood viscosity is initially compensated by increased cardiac output. However, cardiac output seldom doubles, and blood viscosity has the potential of decreasing

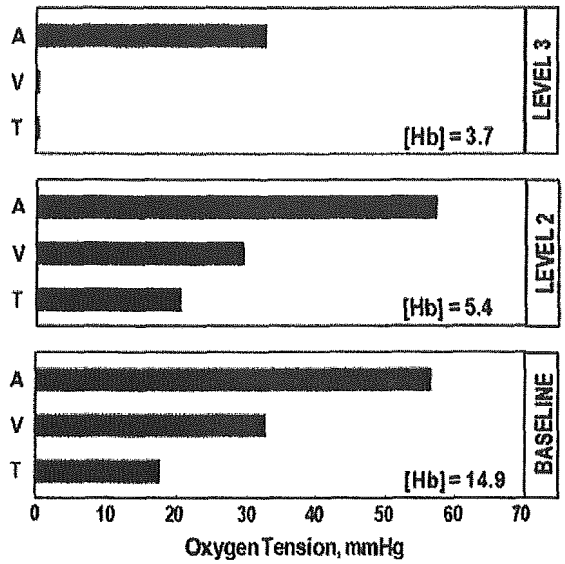

Figure 1 Distribution of oxygen in arterioles (A) and venules (V) in the tissue (T) in the awake hamster window model as a function of total hemoglobin (Hb, grams per deciliter). Normovolemic hemodilution was achieved using dextran 70 kDa. It is apparent that a loss of the order of two thirds of red blood cells has no influence on microcirculatory pO_2 of this model. (see color insert)

to 25 percent of normal upon further reductions of hematocrit, leading to the significant lowering of functional capillary density (FCD, capillaries with red blood cell transit), a condition detrimental to survival in hemorrhagic shock.

Although capillaries do not appear to be the determinant structure for the supply of oxygen in some tissues, the maintenance of FCD in shock is a critical parameter in determining outcome independently of tissue oxygen tension (pO_2), suggesting that extraction of metabolic by-products may be as critical to a capillary function as oxygenation. The relationship between FCD and survival has been demonstrated in experimental analysis of conditions during prolonged hemorrhagic shock, where the principal microvascular functional difference between survivors and nonsurvivors was that survivors maintained FCD above a threshold of about 40 percent of that present in the normal organism; there were no other significant differences between groups [1].

The blood viscosity threshold that causes the decrease in FCD appears to coincide with the decision to transfuse blood. In other words, the transfusion trigger may also be a viscosity trigger; therefore results obtained with blood transfusions may also be achieved by increasing plasma viscosity. Thus use of red blood cells solely for the purpose of increasing blood viscosity may be avoided by introducing a material that increases plasma viscosity in the circulation. In this scenario blood viscosity resulting from the balance between the diminished red blood cell concentration and the increased plasma viscosity leads to the maintenance of vascular resistance. Tsai et al. [2] explored this phenomenon by inducing extreme hemodilution with low-viscosity dextran 70 kDa. At 11 percent hematocrit, FCD and microvascular flow were significantly reduced from control. However, when plasma viscosity was maintained above 2 cP by the introduction of high-viscosity dextran 500 kDa, FCD was maintained near to control values, and microvascular flow increased significantly above control, though hematocrit was 11 percent. This effect was not found if extreme hemodilution was performed with the Biopure product Oxyglobin, even at a total blood hemoglobin content of 6.7 g Hb/dL. High-viscosity plasma also caused blood flow to increase significantly above nonhemodiluted values because of the release of shear-dependent generated endothelial relaxing factors.

Counterintuitive Rheological Findings

High-viscosity plasma restores mean arterial blood pressure (MAP) in hypotension without vasoconstriction. Systemic blood viscosity depends on hematocrit squared; thus viscous losses in major vessels of the circulation can be minimized even when plasma viscosity is increased, shifting pressure and pressure gradients from the systemic to the peripheral circulation. Reduced blood viscosity decreases shear stress and the release of vasodilators, causing vasoconstriction, which negates the benefit of reducing the rheological component of vascular resistance. Vascular resistance depends on the first power of blood viscosity and the 4th power of vessel radius. Therefore reducing blood viscosity with low viscosity plasma might decrease flow and oxygen delivery to the tissue if there is an associated vasoconstrictor stimulus. However, increased flow and/or increased viscosity augments shear stress on the endothelium since the elevation of plasma viscosity causes sustained NO-mediated dilatation in the hamster muscle microcirculation, supporting this interpretation. Enhancement of shear stress on the vessel wall results in the release of prostacyclin and NO from the vascular endothelium. Endogenous NO release reduces total peripheral resistance during moderate hemodilution. Increased wall shear stress following increased blood flow induces vasodilation [3] showing that the link between shear stress and vasodilatation is well established.

Experimental verification of the beneficial effects of high-viscosity plasma during hemodilution in the microcirculation is evidenced by effects on FCD, perfusion and vasodilatation. More recently it was demonstrated by Cabrales et al. [4] that an increase in capillary pressure is the principal mechanical event that governs the effects due to perfusion with high-viscosity plasma.

Hemodilution, Blood Viscosity, and Vasoactivity

It is now apparent that low viscosity molecular hemoglobin solutions lower FCD independently of the intrinsic

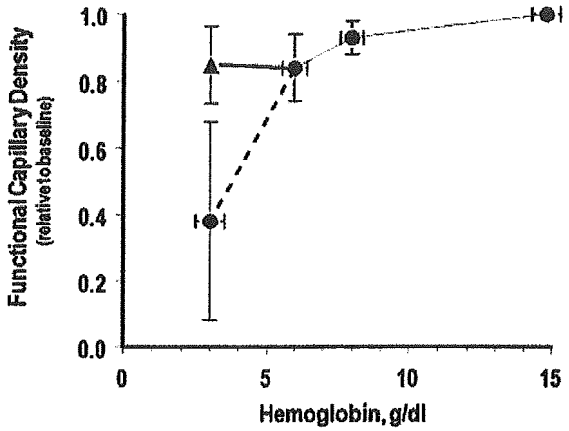

Figure 2 Changes of FCD following isovolemic hemodilution with low- and high-viscosity plasma expanders. ● Hemodilution with dextran 70 kDa maintains FCD up to a hematocrit (hemoglobin) that is 40 percent of normal. Further hemodilution with the same low-viscosity diluent causes the fall of FCD to pathologically low levels. ▲ Continuation of hemodilution with dextran 500 kDa after reaching 40 percent of normal hemoglobin with hemodilution with dextran 70. FCD is maintained to normal levels by the increased plasma viscosity. Redrawn from Tsai et al. [2].

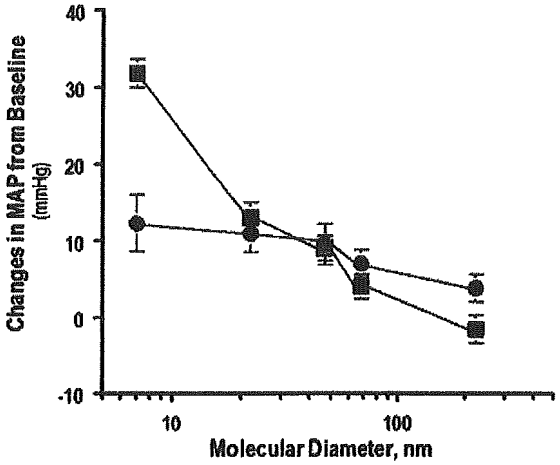

Figure 3 Changes in mean arterial blood pressure after a 5 percent by volume (5 Hb g/dL) topload infusion of free hemoglobin solutions of different molecular diameters and vesicle encapsulated hemoglobin. ■ Pressor effect after infusion. ● Pressor effect 3 hours after infusion. Redrawn from Sakai et al. [5]. (see color insert)

vasoactive properties of the hemoglobin molecule because they cause a significant decrease in blood viscosity after reaching the transfusion trigger. An additional factor attendant to the restoration of blood volume upon reaching the transfusion trigger with a plasma-like viscosity fluid is that this process brings the organism to near extreme hemodilution conditions, characterized by decreased shear stress on the endothelium, lowering the production of endothelial-derived vasodilators. Increasing plasma viscosity to about 2.0 to 2.5 cP increases shear stress and the production of vasodilators, which breaks up the vicious circle caused by extreme hemodilution, compensatory vasoconstriction and low viscosity.

Experimental results shown in Figure 2 show that the maintenance of FCD is not directly linked to oxygen delivery, but to mechanical factors related to the viscosity of the perfusion fluid and the production of vasodilators by mechanotransduction in the endothelium. Therefore an acellular oxygen carrier should maintain plasma viscosity above a specific threshold, while ensuring that overall blood viscosity does not exceed normal values.

Low blood viscosity can be compensated for by hemoglobin solutions with high viscosity. This can be achieved by mixing the hemoglobin molecule with a viscogenic material such as hydroxyethyl starch (HES) at suitable concentrations, or by modifying the hemoglobin molecule to produce an intrinsically viscous solution by increasing its molecular dimension. This latter process can be implemented by polymerization or conjugation with various molecules such as starch and polyethylene glycol, as described by Sakai et al. [5], who showed that the pressor effect is inversely related to molecular size (Figure 3). Hemoglobin molecules with these and several other beneficial features are polyethylene glycol conjugated hemoglobin molecules [6].

The Vasoconstrictive Effect of Hemoglobin

Natural hemoglobin molecules are presumed to be vasoconstrictive because of their ability to scavenge NO. However, recent experimental evidence shows that whereas NO binding is virtually identical for most hemoglobin molecules [7], the vasoconstrictive effect is not, being essentially absent in polyethylene glycol modified hemoglobins and in some very large hemoglobin polymers.

NO is produced by the endothelium as a result of shear stress and other processes. The affinity of the hemoglobin molecule for NO is due to the physical similarity between NO and O_2. Thus in general, hemoglobins with high affinity for O_2 generally also have a high affinity for NO, and vice versa. The production of genetically modified hemoglobins that appear to have little affinity for NO, while maintaining a normal affinity for O_2, may challenge this generalization; however, the fact remains that interfering with NO production with administration of L-arginine methyl ester hydrochloride (L-NAME) or scavenging NO with cell free unmodified hemoglobin causes the constriction of aortic rings, and an increase in blood pressure in experimental subjects.

The concept that hemoglobin extravasation and its location between the endothelium and smooth muscle is the principal factor causing hypertension and vasoconstriction is also questionable because the extravasated molecule will eventually saturate. In fact the presence of a NO-avid mole-

cule in plasma is sufficient to distort the diffusion field of NO from the endothelium, whereby hemoglobin does not need to extravasate to be vasoconstrictive.

NO scavenging does not provide a consistent explanation for the pressor effect of free hemoglobin in the circulation that is applicable to the different hemoglobin modifications. The lack of correlation between pressor responses and NO scavenging characteristic of hemoglobin molecules led McCarthy et al. [8] to propose that hypertension following the introduction of molecular hemoglobin in the circulation is caused by a mechanism related to the process of facilitated diffusion of oxyhemoglobin. According to this hypothesis the presence of molecular hemoglobin causes an additional flux of oxygen in the plasma layer due to the diffusion of oxyhemoglobin. Although the diffusion constant of hemoglobin is low, the amount of oxygen carried is large because hemoglobin binds a large amount of oxygen. The net result of this process is that a comparatively small concentration of molecular hemoglobin augments oxygen transfer to the vessel wall, leading to a hyperoxia signal, and consequently a vasoconstrictive response.

In vivo, peripheral vascular resistance is autoregulated at the level of the arterioles by a mechanism that senses oxygen tension, producing vasodilatory signals when blood and tissue po_2 is low, and vice versa. This conceptualization is supported by the finding that large hemoglobin molecules are not vasoactive, although they carry oxygen. As an example, poly (ethylene glycol) (PEG) surface decorated hemoglobins (PEG-Hb) have consistently been shown to be vasoinactive. These molecules have a large volume because of the water bound by PEG. Since the diffusion constant is inversely proportional to molecular radius, it can be shown that PEG-Hb has a diffusion constant that is about one fifth that of the native hemoglobin.

Experimentation with different levels of hemoglobin surface decoration show that vasoinactivity may be partially related to the degree to which the surface of the hemoglobin molecule is shielded by the water–PEG combination [6]. This phenomenon suggests that free hemoglobin may also cause a pharmacological effect mediated at the surface of the endothelium, and that conjugation of hemoglobin with PEG may produce a shield that prevents this process.

The vasoconstrictive effects of molecular hemoglobin may have several components that sometimes reinforce each other. When blood viscosity becomes too low, there is a reflex vasoconstriction that attempts to maintain perfusion pressure, a phenomenon independent of blood oxygen-carrying capacity. Oxygen regulation plays a crucial role since the arteriolar walls and the tissue sense both the rate of oxygen delivery from the red blood cell column and local po_2. When molecular hemoglobin is present in plasma, there is a significant additional flux of oxygen to the arteriolar wall by facilitated diffusion, a process enhanced with right-shifted oxygen dissociation hemoglobin molecules. NO scavenging can also be a factor that may be balanced by increased NO (and/or prostacyclin) production resulting from elevated shear stress caused by high-viscosity hemoglobin molecules. Furthermore, considering that modest amounts of small molecular hemoglobin can elicit a pressor response, a pharmacological effect due to "naked" small hemoglobin molecules in the circulation may also be present.

Vasoconstriction limits perfusion and decreases FCD. Although healthy organisms could probably compensate for moderate hypertensive episodes leading to corresponding decreases in FCD, these same episodes may place the organism in jeopardy if they are superposed to other vasoconstrictive stimuli, such as those inherent to hemorrhagic shock. Conversely, high plasma viscosity is critical in resuscitation, as an OCPE is administered in conditions of extreme hemodilution because there is no need for using these products prior to reaching the transfusion trigger.

Optimal Oxygen Disassociation Properties

The development of oxygen carriers has implicitly assumed that the oxygen dissociation curve should be right shifted, thus facilitating the release of oxygen. This approach does not consider the longitudinal gradient of oxygen tension in the circulation, whereby a right-shifted dissociation curve favors oxygen unloading from small arteries and arterioles. Hemodilution with hemoglobin-filled vesicles of different p50 in the hamster window chamber model has shown that improved tissue oxygenation is obtained when this parameter is 16 mmHg, instead of 34 mmHg (Department of Polymer Chemistry, Waseda University, Tokyo, Japan). PEG-conjugated hemoglobin (Hemospan, 4% Mal-PEG hemoglobin) produced by Sangart (San Diego, CA), with a p50 of 5 mmHg, used at low concentration in hemodilution maintains FCD and positive acid–base balance.

This apparent paradox may be understood by analyzing the distribution of oxygen in the microcirculation as shown in Figure 4, where oxygen tension in the microcirculation in normal conditions has a baseline tissue po_2 level of 22 to 24 mmHg (which appears to be common for most tissues). It is notable that although oxygen is regulated to achieve this partial pressure in the tissue, anaerobic metabolism occurs when tissue po_2 is below 2.4 to 2.9 mmHg.

A possible rationale for the high po_2 tissue regulation is that the organism has excess oxygen-carrying capacity, not only as a requirement for extreme efforts, but also for compensation of oxygen delivery inhomogeneity in the microcirculation. The effect of this inhomogeneity becomes apparent in considering the variability of oxygen partial pressure distribution in the hamster window chamber, which is of the order of ± 4 mmHg. This variability is a consequence of the quasirandom distribution of the transport properties of the microcirculation, and therefore intrinsic to any level of tissue oxygenation. In conditions of extreme hemodilution tissue po_2 decreases to 3 to 5 mmHg; thus, if

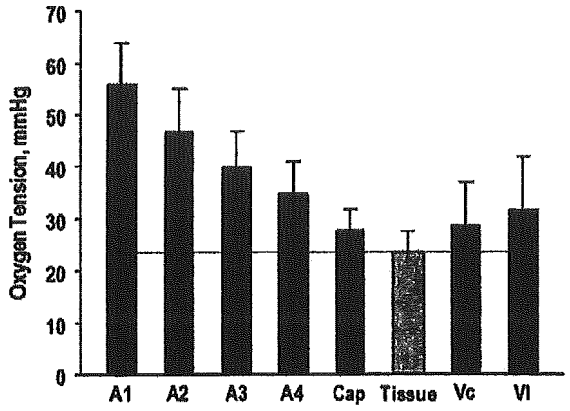

Figure 4 Distribution of pO_2 in the microvessel of the microcirculation of the hamster window preparation as a function of the arteriolar order of branching (As) and the venular order of branching (Vs). It is apparent that tissue pO_2 is narrowly regulated to a value in the range of 22 to 24 mmHg, which is significantly higher than the level associated with anaerobic metabolism.

the same variability is present, there is a significant amount of tissue that is anoxic. In the presence of a fraction of the oxygen-carrying capacity that can only be released at very low pO_2s, a form of targeted oxygen delivery, the effects of this variability will be nullified, ensuring that all the tissue, even at low pO_2 is oxygenated above the anaerobic threshold.

Tissue pO_2 levels that may be considered harmful could, in fact, be quite safe if it were possible to eliminate the inherent variability of oxygen delivery shown by the variability of tissue pO_2. A small quantity of a low-p50 hemoglobin oxygen carrier in the circulation accomplishes this because it delivers oxygen only to portions of the tissue where the anoxic threshold is passed, while the presence of even significant amounts of right-shifted hemoglobin would have no effect since most of the bound oxygen would be unloaded in oxygenated regions.

Cross-linked or polymerized hemoglobins developed so far have a high p50, presumed to be beneficial since it facilitates oxygen unloading. However, pO_2 in the microcirculation is regulated so that there is a significant decrease in oxygen tension from the systemic circulation to the capillaries, which typically have a pO_2 of about 30 mmHg. At this p50 half of the blood oxygen is delivered by arterioles in normal conditions; however, if the p50 of the OCPEs is above this value, as in the case of Oxyglobin (p50 = 54 mmHg), most of the oxygen in the blood should be delivered by the arterioles if this material were to replace blood. These vessels extract a significant amount of oxygen from the circulation while consuming a major portion of this oxygen flux, thus increasing their oxygen supply increases tissue oxygen inhomogeneity, which is further aggravated by the vasoconstrictor autoregulatory response already discussed.

Oxygen-Carrying Capacity

Measurements of pO_2 in the microcirculation utilizing the technique of phosphorescence oxygen quenching show that when hemodilution carried out to a total hemoglobin content in red blood cells of 5.6 g/dL, then tissue oxygen is somewhat higher than normal but not statistically significant. The required oxygen-carrying capacity can also be obtained by a simple calculation that relates the whole-body oxygen consumption and cardiac output, which yields a nearly identical number for the organism at rest. Therefore, in principle, the oxygen-carrying capacity of an OCPE does not need to reproduce the value for normal blood and can be significantly lower.

Colloid Osmotic Pressure

It is generally assumed that a blood substitute should have a colloid osmotic pressure similar to that of blood and in the range of 20 to 25 mmHg; however, several plasma expanders have zero colloid osmotic pressure (saline, Ringer's lactate) and small-volume resuscitation utilizes fluids with very high osmotic properties. To date there is no definitive answer on what is the osmotic and/or oncotic property that is most appropriate, and in all probability this is a variable that depends on the type of blood loss to be corrected. Resuscitation with noncolloidal fluids leads to tissue edema. Conversely fluids with high colloidal and osmotic pressures cause tissue fluid to come into the vascular compartment, thus decreasing the amount of fluid to be administered. Most conditions of hemorrhage are associated with endothelial edema, which has been demonstrated to be rapidly reversed upon the introduction of hyperosmotic and hyperoncotic fluids. Volume expansion fluids such as hydroxyethyl starch have relative high colloid osmotic pressures, typically in the range of 30 to 50 mmHg depending on formulation. Small molecule hemoglobin–based OCPEs have their oncotic pressure adjusted to be that of plasma, but PEG-hemoglobin modified OCPEs tend to have higher oncotic pressures.

Synthesis of an Effective Oxygen-Carrying Plasma Expander

An OCPE based on the preceding concepts is a fluid with properties fundamentally different from those of blood, since it has low oxygen-carrying capacity, p50 is low and in the neighborhood of 5 mmHg, viscogenic properties are such that when introduced into the circulation plasma viscosity should be of the order of 2.0 to 2.5 cP, and colloidal osmotic pressure can be high. A fluid with these properties can be obtained by conjugating hemoglobin with PEG, and various formulations have been tested in both animal experiments and human trials with excellent results. Notably this

formulation is vasoinactive, and its NO-scavenging characteristics do not appear to be relevant since these fluids have the same NO binding constant as other vasoactive formulations that are vasoactive [7].

These fluids are in some cases more effective than blood because they are designed to maintain FCD, which is as necessary as restoring tissue oxygenation for the recovery from blood losses. Because in the foreseeable future OCPEs will use human hemoglobin, these fluids are practical: Their hemoglobin content is low, and more than two units of blood equivalent unit of resuscitation fluid can be obtained from one unit of blood. Finally, this low oxygen-carrying capacity is practical and safe because it yields a significant improvement of microvascular function.

Experimental Evidence

The effectiveness of different resuscitation modalities was tested experimentally in studies of extreme hemodilution and hemorrhagic shock in the microcirculation of the hamster chamber window model, which allows microcirculatory monitoring in the awake condition for a period up to 1 week, after the effects of the surgical intervention have subsided. Extreme hemodilution was chosen because in most instances, lowering systemic hematocrit to 50 percent of baseline with a suitable plasma expander does not alter microvascular hemodynamics and transport in our experimental model. Animals were hemodiluted to 60 percent of normal with dextran 70 kDa, and further hemodiluted to a final hematocrit of 11 percent using the different products simulating blood losses initially remedied with conventional plasma expanders, which upon passing the transfusion trigger are corrected with an oxygen-carrying blood substitute.

A compendium of findings in extreme hemodilution to 50 percent of normal with dextran 70 kDa and further hemodilution to a final hematocrit of 11 percent with the different products is shown in Figure 5, including results obtained with PEG-Hb vesicles developed at Waseda University, Tokyo, using a somewhat different protocol where extreme hemodilution was achieved with a continuous exchange of a hemoglobin vesicle suspension. FCD is shown as a function of blood base excess, which represents systemic conditions and suggests the definition of *critical functional capillary density* as the value for this parameter at which base excess is no longer sustained and drops following modest reductions of total blood hemoglobin, that is, in the neighborhood of a 50 percent FCD reduction. The most important result is that normal base excess is obtained with total blood hemoglobin of 5 percent, if 1 percent of this is Mal-PEG-Hb—a result not found with other OCPEs.

Extreme hemodilution is not a clinically relevant procedure and serves only to study basic mechanisms. A clinically relevant test is to rescue a subject in hemorrhagic shock. Studies were therefore conducted to determine the effects of resuscitation with blood, starch, and Mal-PEG-Hb in a conventional 50 percent bleed shock protocol. The animals were resuscitated after 1 hour without any additional volume manipulation using shed blood, HES, and Mal-PEG-Hb with 25 percent of the blood volume. The results, shown in Figure 6, indicate that Mal-PEG-Hb is superior to both HES and blood in reestablishing microvascular function. Concurrently it was found that base excess was higher in the Mal-PEG hemoglobin-resuscitated animals than in the blood-resuscitated animals. An explanation for these findings is that low p50 hemoglobin targets oxygen delivery of oxygen to only the anoxic tissue.

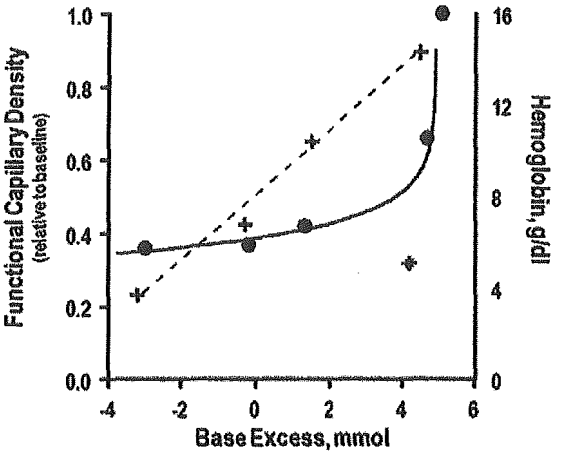

Figure 5 Relationship between total circulating hemoglobin and base excess, and FCD and base excess, for different hemoglobin modifications and concentrations, including hemoglobin vesicles, in normovolemic hemodilution experiments. The data marked ● shows the relationship between FCD and base excess, showing that MAL-Peg-Hb (▼) yields high FCD and base excess at low hemoglobin concentrations. It is apparent that base excess is a direct function of hemoglobin concentration (+) with the exception of MAL-Peg-Hb (✤), which presents normal base excess at a very low total hemoglobin content. (see color insert)

An extreme hemorrhage study was performed with Mal-PEG-Hb in which rats were 50 percent exchange transfused before hemorrhage with either αα-cross-linked hemoglobin, or 4 percent Mal-PEG hemoglobin (Figure 7). These animals were then subjected to a continuous exponential bleed (1 hour, 60 percent of blood volume) whereby at the end of the second hour after the start of bleeding 50 percent of the control animals succumbed. In these experiments it was found that at the end of one hour all animals that received Mal-PEG hemoglobin before hemorrhage survived, while all of those receiving αα-cross-linked hemoglobin did not survive.

Summary and Conclusions

The revision of microvascular physiology related to modifying basic transport properties of blood such as plasma viscosity, p50, and hemoglobin concentration shows

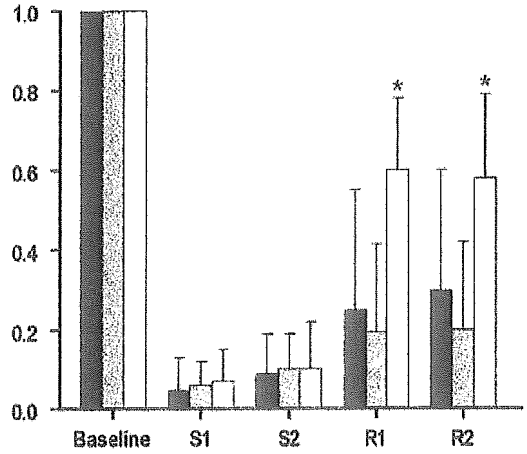

Figure 6 Recovery of FCD during resuscitation from 1 hour hemorrhagic shock with identical volumes of shed blood (*black bars*), 5 percent HES (*shaded bars*), and 4 percent MAL-Peg-Hb (*white bars*). S1 and S2 initial and final conditions during the shock period. R1, Recovery immediately after resuscitation; R2, 1 hour after resuscitation. $p < 0.05$ relative to shed blood and HES.

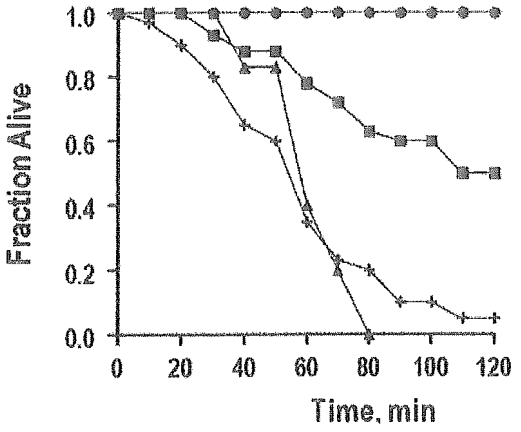

Figure 7 Controlled bleeding in rats that are 50 percent exchange transfused with MAL-Peg-Hb (●), αα-cross-linked hemoglobin (▲), and a polymerized hemoglobin (+), versus controls (■) with no treatment. The study was designed so that 50 percent of the untreated (not transfused controls) would survive 120 minutes.

that blood or a bloodlike fluid may not be the optimal oxygen-carrying volume-restoring fluid. A critical parameter for either oxygen-carrying or noncarrying blood replacements is their viscosity, which is a factor in maintaining capillary flow.

Analysis of the microvascular consequences of changing blood rheological conditions and particularly plasma shows that low plasma viscosity is not of universal benefit. Patients following trauma, peripheral arterial occlusive disease, and acute myocardial infarction have elevated plasma viscosity, a condition presumed to be pathological. However, there are situations where increased viscosity may be a protective or beneficial mechanism.

Plasma expanders are not used after reaching the transfusion trigger because the reduction of blood oxygen-carrying capacity beyond this point is assumed to jeopardize tissue oxygenation, according to the systemic evaluation of the organism portrayed by blood gases. Conditions in the microcirculation and local microscopic tissue environment when the reduction of red blood cells is extended beyond the transfusion trigger have not been consistently explored and presently show that oxygen-carrying capacity is not the major factor in determining tissue survival.

Studies show that the transfusion trigger is also the limit for the organism to adapt to low blood viscosity in acute conditions; thus *the conventional transfusion trigger is also a viscosity trigger*. Since the administration of a molecular oxygen carrier is physically similar to continuing fluid therapy after reaching the transfusion trigger, the maintenance of FCD requires the increase of plasma viscosity which through shear stress–dependent mechanisms operating in the endothelium ensures the maintenance of optimal microvascular function. Oxygen-carrying capacity is exhausted upon red blood cell (or hemoglobin) losses that are significantly greater than those represented by the transfusion trigger. However, these losses of oxygen-carrying capacity do not need to be compensated on a one-to-one basis, if microvascular function (i.e., FCD) is maintained and an oxygen carrier is introduced only to deliver oxygen to anoxic tissue regions. This approach ensures a uniform maintenance of the whole organism above the anaerobic threshold, while limiting the amount of oxygen carrier needed to maintain metabolism. Thus the combination of maintenance of microvascular function and targeted oxygen delivery is the primary determinant of an efficacious human hemoglobin-based blood substitute that is more effective than blood in acute conditions and that also expands the available blood supply, since a unit of blood yields more than two units of surrogate blood.

Glossary

Functional capillary density: Number of capillaries in a unit volume of tissue that presents the passage of red blood cells. This parameter is experimentally determined by measuring the length of red blood cell–perfused capillaries in a microscopic field of view.

Microvascular function: A combination of parameter including flow, number of open capillaries, intact vascular permeability, and level of vessels tone that allows for the proper interaction between blood and tissue at the microscopic level.

Oxygen-carrying capacity: The amount of oxygen in milliliters at standard atmospheric conditions and temperature contained in a fluid.

p50: Partial pressure of oxygen at which hemoglobin is 50 percent saturated with oxygen.

Plasma expander: A fluid used to restore circulatory volume when oxygen-carrying capacity is adequate.

Transfusion trigger: Level of blood hemoglobin at which the decision is made to introduce red blood cells into the circulation in order to restore oxygen-carrying capacity.

Vasoactivity: Inherent property of compounds that cause vasoconstriction and the elevation of systemic blood pressure.

Acknowledgments

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References

1. Kerger, H., Saltzman, D. J., Menger, M. D., Messmer, K., and Intaglietta, M. (1996). Systemic and subcutaneous microvascular pO_2 dissociation during 4-h hemorrhagic shock in conscious hamsters. *Am. J. Physiol.* **270**, H827–H836. *Reports the basic data showing the direct correlation between survival and maintenance of functional capillary density.*
2. Tsai, A. G., Friesenecker, B., McCarthy, M., Sakai, H., and Intaglietta, M. (1998). Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skin fold model. *Am. J. Physiol.* **275**, H2170–H2180. *Experimental demonstration that high viscosity plasma in extreme hemodilution maintains microvascular function and systemic conditions, an effect that is not present at an identical reduction of hematocrit (oxygen-carrying capacity) when plasma viscosity is normal.*
3. Smiesko, V., and Johnson, P. C. (1993). The arterial lumen is controlled by flow-related shear stress. *News Physiol. Sci.*, 34–38.
4. Cabrales, P., Tsai, A. G., and Intaglietta, M. (2004). Microvascular pressure and functional capillary density in extreme hemodilution with low and high plasma viscosity expanders. *Am. J. Physiol. Heart Circ. Physiol.* **287**, H363–H373. *Experimental demonstration that capillary blood pressure and functional capillary density are directly related. In extreme hemodilution increased plasma viscosity elevates capillary pressure at reduced overall oxygen delivery. By comparison, hemodilution to the same hematocrit, but increased blood hemoglobin following the introduction of a low-viscosity vasoactive hemoglobin solution, lowers capillary pressure and FCD.*
5. Sakai, H., Hara, H., Yuasa, M., Tsai, A. G., Takeoka, S., Tsuchida E., and Intaglietta, M. (2000). Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol.* **279**, H908–H915.
6. Manjula, B. N., Tsai, A. G., Intaglietta, M., Ho, C., Malavalli, A., Vandegriff, K., Winslow, R. M., Friedman, J. M., Smith, P. K., and Acharya, S. A. (2003). Thiolation mediated, maleimide-chemistry based pegylation of Hba: Design, preparation and characterization of (PEG5K)6-hba, a new non-hypertensive Hb-based oxygen carrier. *Bioconjug. Chem.* 2003.
7. Rohlfs, R. J., Brunner, E., Chiu, A., Gonzales, A., Gonzales, M. L., Magde, D., Magde, M. D., Jr., Vandegriff, K. D., and Winslow, R. M. (1998). Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. *J. Biol. Chem.* **273**, 12128–12134. *Direct demonstration that vasoactivity and the induction of hypertension are not dependent on NO scavenging, since hemoglobin solutions with the same NO binding capacity cause a range of responses, varying from no effect on blood pressure found with pegylated hemoglobin to a maximal increase in blood pressure found with $\alpha\alpha$-hemoglobin.*
8. McCarthy, M. R., Vandegriff, K. D., and Winslow, R. M. (2001). The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: Implications for the design of hemoglobin-based oxygen carriers. *Biophys. Chem.* **92**, 103–117.

Further Reading

Kobayashi, K. (2004), *Artificial Oxygen Carrier: Its Frontline*, Vol. 12. Tokyo: Springer-Verlag.

Rudolph, A. S., Rabinovich, R., and Feurestein, G. Z. (1998). *Red Blood Cell Substitutes. Basic Principles and Clinical Applications.* New York: Marcel Dekker.

Tsuchida, E. (1998). *Blood Substitutes: Present and Future Perspectives.* Amsterdam: Elsevier Science.

Winslow, R. M., Vandegriff, K. D., and Intaglietta, M. (1995). *Blood Substitutes. Physiological Basis of Efficacy.* Boston: Birkhäuser.

Winslow, R. M., Vandegriff, K. D., and Intaglietta, M. (1996). *Blood Substitutes. New Challenges.* Boston: Birkhäuser.

Winslow, R. M., Vandegriff, K. D., and Intaglietta, M. (1997). *Advances in Blood Substitutes. Industrial Opportunities and Medical Challenges.* Boston: Birkhäuser.

Biographies

Amy G. Tsai obtained her Ph.D. in bioengineering at the University of California, San Diego, where she is currently a Senior Research Scientist. She is widely recognized for her findings on oxygen consumption by the microvasculature and the development of high-viscosity plasma expanders. She is an expert in mathematical modeling, measuring methods for the in vivo study of the microcirculation and small animal experimentation.

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Prof. Marcos Intaglietta received his Ph.D. in applied mechanics from the California Institute of Technology in Pasadena and developed his academic career at the University of California, San Diego, where he is one of the founders of the bioengineering program and department. His specialty is the study of transport phenomena in the microcirculation and the development of blood substitutes. He has developed and implemented most of the methods presently used for the study of the microcirculation.

Variant tricarboxylic acid cycle in *Mycobacterium tuberculosis*: Identification of α-ketoglutarate decarboxylase

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Mycobacterium tuberculosis **(Mtb) has adapted its metabolism for persistence in the human macrophage. The adaptations are likely to involve Mtb's core intermediary metabolism, whose enzymes have been little studied. The tricarboxylic acid cycle is expected to yield precursors for energy, lipids, amino acids, and heme. The genome sequence of Mtb H37Rv predicts the presence of a complete tricarboxylic acid cycle, but we recently found that α-ketoglutarate dehydrogenase (KDH) activity is lacking in Mtb lysates. Here we showed that citrate synthase, aconitase, isocitrate dehydrogenase, fumarase, malate dehydrogenase, and succinate dehydrogenase, but not KDH, are present, raising the possibility of separate oxidative and reductive half-cycles. As a potential link between the half-cycles, we found that Rv1248c, annotated as encoding SucA, the putative E1 component of KDH, instead encodes α-ketoglutarate decarboxylase (Kgd) and produces succinic semialdehyde. Succinic semialdehyde dehydrogenase activity was detected in Mtb lysates and recapitulated with recombinant proteins GabD1 (encoded by Rv0234c) and GabD2 (encoded by Rv1731). Kgd and GabD1 or GabD2 form an alternative pathway from α-ketoglutarate to succinate. Rv1248c, which is essential or required for normal growth of Mtb [Sassetti, C., Boyd, D. H. & Rubin, E. J. (2003) *Mol. Microbiol* 48, 77–84] is the first gene shown to encode a Kgd. Kgd is lacking in humans and may represent a potential target for chemotherapy of tuberculosis.**

succinic semialdhehyde | succinic semialdhehyde dehydrogenase

Mycobacterium tuberculosis (Mtb), the leading cause of death from a single bacterial species, severely impacts global health and threatens further havoc with the spread of drug resistance (1). Chemotherapy directed against new classes of targets is an urgent need (2). Unfortunately, knowledge is scant regarding the metabolism of Mtb during its residence in host cells and organs. Transcriptional adaptations of the bacillus in those sites imply that some of the environments faced by Mtb in the host are hypoxic, carbon-poor, oxidative, and nitrosative (3–6).

Part of the difficulty in delineating Mtb's metabolic profile during infection is the meagerness of biochemical knowledge pertaining to its core metabolic processes, much of which has been tentatively inferred from bioinformatics. Several metabolic enzymes are linked with persistence and antioxidant defense of Mtb. Isocitrate lyase (Icl) and glyoxylate dehydrogenase, two enzymes of the glyoxylate shunt, are activated during adaptation to microaerophilic conditions (7), and *icl* expression is enhanced during infection of macrophages (8). Mtbs deficient in *icl* were attenuated for survival in activated macrophages *in vitro* and in lungs of mice (9). Two constituents of pyruvate dehydrogenase (PDH) serve also as components of one of the antioxidant and antinitrosative systems in Mtb; these are lipoamide dehydrogenase (Lpd; Rv0462) and dihydrolipoamide acyltransferase (DlaT; Rv2215; formerly SucB). Lpd and DlaT interact with two other proteins to constitute a four-protein, NADH-dependent peroxidase and peroxynitrite reductase. The two additional proteins are alkylhydroperoxide reductase subunit C (AhpC) and the thioredoxin-like protein coexpressed with it, AhpD (10). This close intersection between core intermediary metabolism and resistance to host-imposed biochemical stress in Mtb directed our attention to Mtb's tricarboxylic acid (TCA) cycle.

The TCA cycle plays essential roles in cell metabolism, providing reducing equivalents for energy generation and biosynthetic reactions, along with precursors for lipids, amino acids and heme. Many bacteria operate variant TCA cycles, reflecting a diversity of metabolic niches and needs (11). The classic cycle favors generation of energy as well as biosynthetic precursors, whereas variants with oxidative and reductive branches, as in many phototropes, lithotrophs and methylotrophs, are more focused on production of biosynthetic precursors (12). The distinguishing feature of the branched variants is their lack of α-ketoglutarate dehydrogenase (KDH). The oxidative branch terminates in production of α-ketoglutarate, whereas the reductive branch leads to succinate. Some bacteria, such as *Escherichia coli*, operate a complete TCA cycle aerobically but can switch under anaerobic conditions to a branched pathway lacking KDH. *Helicobacter pylori*, which thrives under microaerophilic conditions, operates a branched TCA pathway, but both branches can be connected by α-ketoglutarate ferredoxin oxidoreductase (13).

The Mtb genome is annotated to encode a complete TCA cycle as well as α-ketoglutarate ferredoxin oxidoreductase (14). However, the annotations are not based on biochemical evidence from Mtb itself. In fact, the only systematic study of TCA cycle enzymes in Mtb dates back to 1962 (15). In that work, PDH and KDH activities were both found to be $NADP^+$-dependent, and KDH was not CoA-dependent. Such unusual patterns of utilization of cofactors call those identifications into question.

Our previous studies (16) suggest that DlaT and Lpd do not conform to their annotations (http://genolist.pasteur.fr/TubercuList) as components of KDH. In fact, Mtb appears to lack KDH, and DlaT and Lpd serve as components of PDH instead. Mtb's PDH activities depend on NAD^+, not $NADP^+$, as measured both in cell extracts and *in vitro* with recombinant proteins. In the present study, we explored the implications of the absence of KDH activity in Mtb. We surveyed the remaining enzymes of the classic TCA cycle and sought additional enzymes that might bridge the oxidative and reductive half-cycles that are potentially left when KDH is absent.

As a starting point, we focused on Rv1248c, whose protein product was annotated as SucA (http://genolist.pasteur.fr/TubercuList) in the expectation that it served as the E1 component

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Abbreviations: Mtb, *Mycobacterium tuberculosis*; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; SSA, succinic semialdehyde; SSADH, SSA dehydrogenase; Kgd, α-ketoglutarate decarboxylase; KPi, potassium phosphate; TPP, thiamine pyrophosphate; KDH, α-ketoglutarate dehydrogenase.

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of KDH. Because the Rv1248c protein does not perform such a function *in vitro* (16), we considered additional actions that it might catalyze with α-ketoglutarate.

Materials and Methods

Bacterial Lysates. Mtb lysates were prepared as described (16). *E. coli*, *Mycobacterium smegmatis*, and *Mycobacterium bovis* bacillus Calmette–Guérin were grown in LB or 7H9 medium at 37°C. Log phase and stationary phase cells were harvested and washed three times in PBS. Cell pellets were resuspended in PBS/1 mM PMSF and broken by beating with glass beads. Lysates were centrifuged twice at 17,900 × *g* for 10 min at 4°C, except that for succinyl CoA synthetase and succinate dehydrogenase assays, lysates were centrifuged once at 2,700 × *g*. Supernatants were used as cell extracts.

Recombinant Proteins. The Rv1248c protein was purified from recombinant *E. coli* as described (16). MtbH37*Rv gabD1* (Rv0234c) and *gabD2* (Rv1731) genes were amplified from genomic DNA by PCR with *Pfu* DNA polymerase using primers containing 5′ NdeI and 3′ NheI sites. PCR products were digested with NdeI and NheI, and ligated with T4 ligase into pET28b vectors digested with the same enzymes. *E. coli* expression strain BL21 (DE3) cells were transformed with pET28b encoding His_6-tagged GabD1 and GabD2 and cultured in 1-liter batches in LB media with 50 μg/ml kanamycin. Protein expression was induced with 1 mM IPTG at 25°C for 4 h. Cells were harvested by centrifugation at 4°C at 5,500 × *g*, resuspended in lysis buffer (1 mM PMSF/30 mM imidazole/25 mM Tris, pH 8.0/500 mM NaCl) and broken in a French press. The soluble fraction of the lysates was loaded on a 5-ml Ni^+ column (equilibration buffer: 30 mM imidazole/25 mM Tris, pH 8.0; washing buffer: 30 mM imidazole/300 mM NaCl/25 mM Tris, pH 8.0; and elution buffer: 250 mM imidazole/300 mM NaCl/25 mM Tris, pH 8.0). Fractions containing recombinant proteins were identified by SDS/PAGE. Protein concentrations were measured by Bradford assay, and proteins were stored at −20°C.

Enzyme Assays. All reactions were performed at room temperature.

KDH. Reaction mixtures (0.5 ml) contained 50 mM potassium phosphate (KPi) (pH 7.0), 0.2 mM thiamine pyrophosphate (TPP), 1 mM $MgCl_2$, 2 mM NAD^+ or $NADP^+$, and 50 μg MtbH37*Rv* lysates. Reactions were initiated by adding 0.165 mM CoA and 1 mM α-ketoglutarate and monitored by following the production of NADH or NADPH at 340 nm. An extinction coefficient of 6,223 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates of NADH or NADPH formation.

Citrate synthase. Reaction mixtures (0.5 ml) contained 50 mM Hepes (pH 8.0), 2 mM EDTA, 100 mM NaCl, 100 μM 5,5′-dithiobis-2-nitrobenzoic acid, and 25 μg of MtbH37*Rv* lysates. Reactions were initiated by addition of 0.2 mM oxaloacetate and 0.14 mM acetyl-CoA, and thionitrobenzene formation was followed. An extinction coefficient of 13,600 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Aconitase. Reaction mixtures (0.25 ml) contained 25 mM Hepes (pH 8.0), 100 mM NaCl, and 50 μg MtbH37*Rv* lysates. Reactions were initiated by adding 0.1 mM *cis*-aconitate and monitored by following the disappearance of *cis*-aconitate at 240 nm. An extinction coefficient of 3,500 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Isocitrate dehydrogenase. Reaction mixtures (0.5 ml) contained 50 mM Hepes (pH 8.0), 0.25 mM NAD^+ or $NADP^+$, 10 mM $MgCl_2$, and 50 μg of MtbH37*Rv* lysates. Reactions were initiated by adding 1 mM isocitrate and monitored by following the production of NADH or NADPH at 340 nm. An extinction coefficient of 6,223 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Succinyl-CoA synthetase. Reaction mixtures (0.5 ml) contained 50 mM KPi (pH 7.0), 100 μM 5,5′-dithiobis-2-nitrobenzoic acid, 250 μM ADP, and 195 μg of MtbH37*Rv* lysates. Reactions were initiated by adding 150 μM succinyl-CoA and monitored by following the formation of thionitrobenzene at 412 nm. Extinction coefficient 13,600 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Succinate dehydrogenase. Reaction mixtures (0.5 ml) contained 50 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 0.18 mM benzyl viologen, and 195 μg of MtbH37*Rv* cell lysates. Reactions were initiated by adding 20 mM fumarate and monitored by following the oxidation of benzyl viologen at 550 nm. An extinction coefficient of 7,800 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Fumarase. Reaction mixtures (0.25 ml) contained 50 mM KPi (pH 7.0) and 50 μg of MtbH37*Rv* lysates. Reactions were initiated by adding 25 mM L-malate and monitored by following the production of fumarate at 250 nm. An extinction coefficient of 1,479 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Malate dehydrogenase. Reaction mixtures (0.5 ml) contained 50 mM Hepes (pH 8.0), 1 mM NADH, and 50 μg of MtbH37*Rv* lysates. Reactions were initiated by adding 400 μM oxaloacetate and monitored by following the consumption of NADH at 340 nm. An extinction coefficient of 6,223 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Succinic semialdehyde dehydrogenase (SSADH). Reaction mixtures (0.5 ml) contained 50 mM Hepes (pH 8.0), 1 mM 2-mercaptoethanol, 2 mM NAD^+ or $NADP^+$, and 50 μg of MtbH37*Rv* lysates. Reactions were initiated by adding 0.1 mM succinic semialdehyde (SSA) and monitored by following the production of NADH or NADPH at 340 nm. An extinction coefficient of 6,223 $M^{-1}\cdot cm^{-1}$ was used to calculate the rates.

Ferricyanide reductase. Ferricyanide reductase was assayed as described (16).

Linked assay of Rv1248c protein and GabD1 or GabD2. Recombinant Rv1248c protein was preincubated with reaction mix A containing 50 mM KPi (pH 7.0), 0.3 mM TPP, 1 mM $MgCl_2$, and 1 mM α-ketoglutarate for 1 h at room temperature. Recombinant GabD1or GabD2 was added to reaction mix B containing 100 mM KPi (pH 7.0), 0.5 mM $NADP^+$, and 1 mM DTT. SSADH activity was followed by measuring the production of NADPH at A_{340} after combining the above two reactions.

Identification of Reaction Product of Rv1248c Protein with α-Ketoglutarate. Recombinant Rv1248c protein was preincubated with reaction mix A (see above) for 1 h. Reaction products were derivatized with 2,4-dinitrophenylhydrazine as described (17) with modifications: 2.5 ml reaction mixture contained 10 mM Tris·HCl (pH 6.5), 0.3 mM TPP, 1 mM $MgCl_2$, and 1 mM α-ketoglutarate. Reaction was initiated by adding 2 μM Rv1248c protein, and 0.5-ml aliquots were removed at time 0, 15, 30, and 60 min. A total of 84 μl of 6.3 mM 2,4-dinitrophenylhydrazine in 3 M HCl was added to each aliquot and the samples incubated for 45 min at 50°C. The derivatized products were extracted three times with 0.5 ml of ethyl acetate, and the combined extracts were evaporated to dryness. The residue was redissolved in 10 μl of ethyl acetate, 5 μl of which was spotted onto a TLC plate (Silica Gel 60/Kieselguhr F_{254}). The plate was developed with 1-butanol saturated with 3% NH_4OH. α-ketoglutarate and SSA (Sigma) were derivatized in the same way and used as standards. For NMR identification, a 0.65-ml reaction mixture contained 50 mM KPi (pH 6.5), 0.3 mM TPP, 1 mM $MgCl_2$, and 5 mM α-ketoglutarate. The reaction was initiated by adding 2 μM Rv1248c protein. Deuterium oxide (D_2O) was added immediately before NMR data collection to a final concentration of 10%. The one-dimensional proton spectra were acquired at 25°C with presaturation of the water resonance. Chemical shifts are quoted relative to external sodium 4, 4-dimethyl-4-silapentane-1-sulfonate at 0 ppm.

Western Blots. MtbH37*Rv* lysates (25 μg) were run on SDS/10% PAGE, transferred to nitrocellulose, and subjected to Western blotting with antiserum (1:10,000) raised by injecting rabbits with purified α-ketoglutarate decarboxylase (Kgd) in incomplete Freund's adjuvant.

Table 1. TCA cycle and related enzyme activities in Mtb H37*Rv*, *M. smegmatis*, *M. bovis* bacillus Calmette–Guérin, and *E. coli* lysates, compared to those published for Mtb (15)

Enzyme	*Mtb* H37Rv V_{max} nmol·min^{-1}·mg^{-1}	*M. smegmatis* V_{max} nmol·min^{-1}·mg^{-1}	*M. bovis* BCG V_{max} nmol·min^{-1}·mg^{-1}	*Mtb* H37Rv V_{max}* nmol·min^{-1}·mg^{-1}	*E. coli* V_{max} nmol·min^{-1}·mg^{-1}
PDH	64	33.4	4.4	3	206
Citrate synthase	405	182	99.2	NM	570
Aconitase	195	770	244.4	154	ND
Isocitrate dehydrogenase	64	233	22.2	87	92
KDH	ND	ND	ND	3	224
Succinyl-CoA synthetase	7.5	25	20.5	NM	20
Succinate dehydrogenase	6.4	47.5	11.5	7†	46.1
Fumarase	548	1,222	1,042	633	279
Malate dehydrogenase	1,149	ND	35.8	2,981	2,229

ND, not detected; NM, not measured.
*Published TCA cycle and related enzyme activities in *Mtb* H37Rv (15).
†Measured by reduction of 2,6-dichlorophenol-indophenol.

Determinations of TCA Cycle Metabolites Concentrations in Mtb Lysates. Protein concentrations of log phase and stationary phase lysates were 4 mg/ml and 7 mg/ml, respectively. Methanol (100 ml) was added to lysates (25 ml), and the mixture was centrifuged (17,000 × *g*) at 4°C for 10 min. The methanol supernatant was diluted 2-fold with distilled water, and a 5-μl aliquot was injected into an Agilent 1100 LC system with a YMC Hydrosphere C18 column connected to an Applied Biosystems/MDS Sciex API 3000 mass spectrometer. Five-milliliter mixtures of calibration standards prepared at concentrations of 1, 10, and 100 mM in distilled water were also injected. Quantification was performed by the absolute calibration method using peak area. Recovery rates were also estimated (Table 3, which is published as supporting information on the PNAS web site).

Estimation of Intracellular Glutamate Concentration. Volume of a bacterial cell was taken as 2×10^{-15} liters (18) or 2.5 μl/mg dry weight (19) assuming that the weight of one cell is 9.5×10^{-13} g and the dry weight of one cell is 2.8×10^{-13} g (20). Alternatively, the volume of a tubercle bacillus was calculated assuming that one cell represents a cylinder with width ranging from 0.3 to 0.6 μm and length from 1 to 4 μm and that lipid represents ≈60% of wet weight (21). Total cell numbers were calculated by using 25-ml cultures at 0.6 OD (logarithmic phase) or 3.0 OD (stationary phase) with the observed relationship that 1 OD unit corresponds to 5×10^8 colony forming units/ml and the assumption that each bacillus is a colony-forming unit. Nonpeptidyl glutamate was measured in lysates with volume 0.7 ml (4.5 mg per ml of protein) from a logarithmic phase culture and 1 ml (7.5 mg per ml of protein) from a stationary phase culture.

Results

TCA Cycle Enzyme Activities in Mtb Lysates. All classical TCA cycle activities other than KDH were detected in Mtb lysates (Table 1). Nor was KDH activity detected in *M. smegmatis* and *M. bovis* bacillus Calmette–Guérin grown on glycerol or glutamate. Specific activities of the other TCA cycle enzymes were comparable to those reported for Mtb in 1962 (15). Thus, it appears that Mtb's TCA cycle is interrupted by a lack of KDH.

Rv1248c Encodes Kgd. We next sought an alternative pathway from α-ketoglutarate to succinate. We began by testing Rv1248c for reactions with α-ketoglutarate other than its dehydrogenation. Recombinant Rv1248c protein was purified to homogeneity as described (16). Immunoblot with antiserum raised against the pure protein documented expression of Kgd in Mtb during both logarithmic growth and stationary phase (Fig. 1*A Left*). Although the Rv1248c protein generated no KDH activity together with DlaT and Lpd (16), it did react with α-ketoglutarate in the presence of TPP and Mg^{2+} as monitored by using ferricyanide as an electron acceptor (Fig. 1*A Right*). The ferricyanide reductase activity of Rv1248c protein (0.8 μmol/min/mg; 109 min^{-1}) was strictly α-ketoglutarate-, TPP-, and Mg^{2+}-dependent, but did not require CoA, NAD^+, or $NADP^+$ (data not shown). The enzyme was specific for α-ketoglutarate, showing no activity with pyruvate. The K_m values for α-ketoglutarate, TPP, and Mg^{2+} were 480 μM, 19 μM, and 196 μM, respectively (Fig. 1 *B–D*), similar to those of the *Euglena gracilis* mitochondrial Kgd (22). Based on the results to be described next, the Rv1248c protein was renamed Kgd.

Given that the foregoing activity of Kgd did not depend on CoA, NAD^+, or $NADP^+$, we hypothesized that Kgd catalyzes an oxidative decarboxylation from five-carbon α-ketoglutarate to four-carbon SSA (molecular mass, 102 Da). TLC demonstrated that the derivatized reaction product formed by incubating Kgd with α-ketoglutarate comigrated with derivatized, authentic SSA (Fig. 2*A*). Formation of SSA was confirmed by one-dimensional proton [^{1}H] NMR spectra (Fig. 2*B*). Two major peaks between 1.5 ppm and 3 ppm represented α-ketoglutarate, and four major peaks were generated from authentic SSA. The reaction mixture of α-ketoglutarate and Kgd generated all six peaks, corresponding to α-ketoglutarate and SSA. The peak between 9 and 10 ppm representing an aldehyde proton was also observed in the reaction samples. The major peak between 4 and 5 ppm represented H_2O.

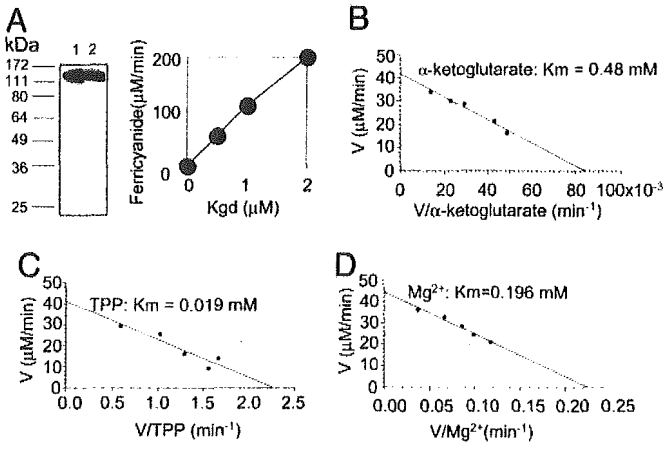

Fig. 1. Expression of native Kgd and activity of recombinant Kgd. (*A*) (*Left*) Immunoblot of lysates (25 μg) of Mtb grown in logarithmic (lane 1) and stationary phase (lane 2) after separation by SDS/10% PAGE, using rabbit antiserum raised against pure recombinant Kgd, which has a calculated Mr of 136 kDa. (*Right*) Ferricyanide reductase activity of recombinant Kgd. (*B–D*) Determination of Kgd Km for α-ketoglutarate (*B*), TPP (*C*), and Mg^{2+} (*D*).

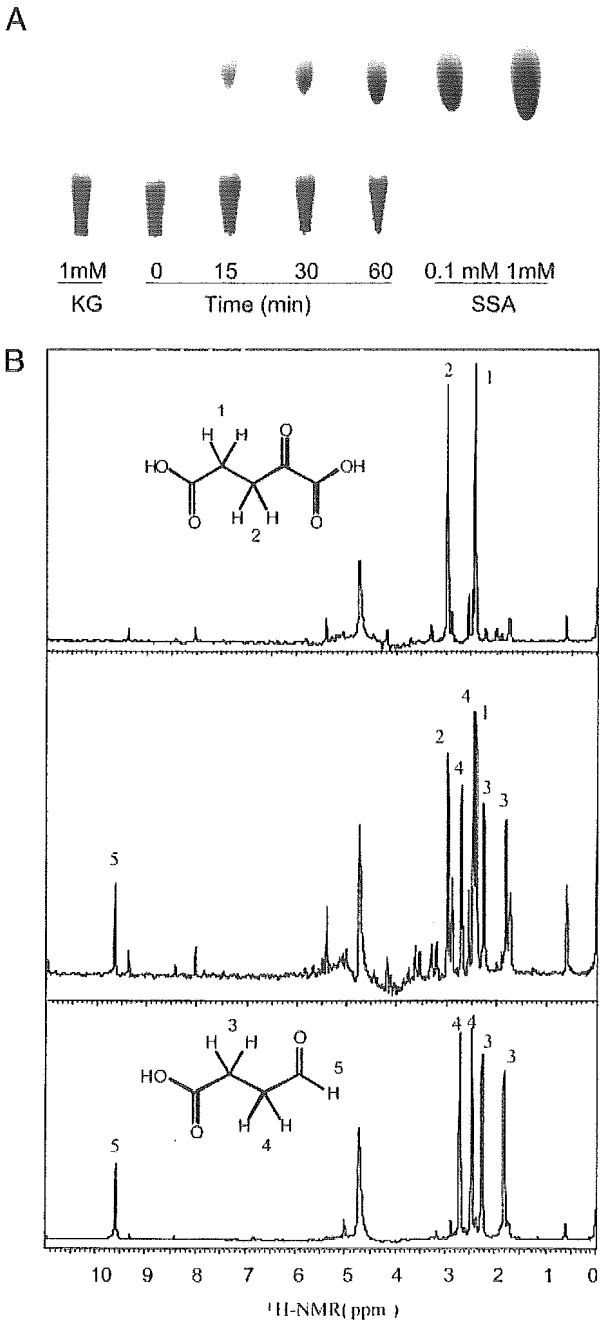

Fig. 2. Kgd catalyzes the reaction from α-ketoglutarate to SSA. (*A*) Identification of the Kgd reaction product by TLC. Products were followed at 0, 15, 30, and 60 min. (*B*) Identification of Kgd reaction product by NMR. [^{1}H]-NMR spectra of samples for reaction mixture with α-ketoglutarate (*Top*), reaction mixture with α-ketoglutarate and Kgd (*Middle*), and authentic SSA control (*Bottom*) are shown. A concentration of 2 μM Kgd was used.

SSADH Activity in Mtb. SSADH, an enzyme of the γ-aminobutyrate shunt, can oxidize SSA to succinate. Because Kgd generated SSA, we hypothesized that Mtb might express SSADH. If so, Kgd and SSADH together could convert α-ketoglutarate to succinate and thereby potentially join the two TCA half-cycles.

Indeed, SSADH activity was detected in lysates of Mtb, *M. smegmatis*, and *M. bovis* bacillus Calmette–Guérin (Table 2). The SSADH activity of Mtb was $NADP^+$-dependent. The specific activities from mycobacteria were comparable to those reported for *Bradyrhizobium japonicum* and potato (17, 23).

Table 2. SSADH activities in Mtb H37*Rv*, *M. bovis* bacillus Calmette–Guèrin (BCG), and *M. smegmatis* lysates compared to those published for *B. japonicum* (17) and potato (23)

	Activity V_{max}, nmol·min^{-1}·mg^{-1}	
Cell lysates	$NADP^+$	NAD^+
Mtb	40.7	4.7
M. bovis BCG	32.1	NM
M. smegmatis	13.3	NM
B. japonicum	9.4	11.1
Potato	NR	7.8

The enzyme activities from Mtb, *M. bovis* BCG, and *M. smegmatis* compared to that published for *B. japonicum* (17) and potato SSADH (23). NR, not reported; NM, not measured.

GabD1 and/or GabD2 were considered the most likely sources of SSADH activity in Mtb. The recombinant proteins were produced in *E. coli* and purified. Both GabD1 and GabD2 displayed $NADP^+$-dependent SSADH activities (Fig. 3*A*). Both proteins could use NAD^+ as a cofactor, but the specific activities were three times lower with NAD^+ as compared to $NADP^+$. The V_{max} of GabD1 was much higher than that of GabD2, and GabD2 is likely to serve physiologically as a dehydrogenase for a different aldehyde(s). The specific activity of GabD1, 0.8 μmol/min/mg protein (32 min^{-1}), was in the range reported for human and rat protein (24).

Combined Reaction of Kgd and GabD1 or GabD2. Next, we tested whether Kgd and either GabD1 or GabD2 together could catalyze a coupled reaction from α-ketoglutarate to succinate. In fact, recombinant Kgd and recombinant GabD1 or GabD2 reduced $NADP^+$ to NADPH in a reaction dependent on α-ketoglutarate, TPP, and Mg^{2+} (Fig. 3*B*).

TCA Cycle Metabolites in Mtb Lysates. Finally, we tried to quantify SSA and TCA cycle intermediates in Mtb lysates. Succinate, fumarate, malate, and glutamate were readily detected in both logarithmic and stationary phase lysates (Table 4, which is published as supporting information on the PNAS web site). Malate and glutamate concentrations were higher in log phase, whereas succinate and fumarate levels remained comparable in log and stationary phases. Glutamate concentrations were 170- to 880- fold higher than concentrations of succinate, fumarate, or malate. We were not able to detect pyruvate, citrate, aconitate, isocitrate, α-ketoglutarate, succinyl-CoA, and SSA under our conditions, which may reflect the poor stability and low recovery of their authentic standards (Table 3).

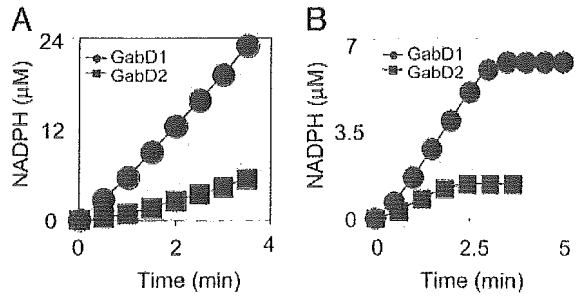

Fig. 3. Kgd together with GabD1 or GabD2 can form a pathway from α-ketoglutarate to succinate in Mtb. (*A*) SSADH activities are encoded by GabD1 (circles) and GabD2 (squares). The amount of each protein used was 0.2 nmol. (*B*) Kgd (2 μM) and GabD1 (0.4 μM) (circles) or Kgd (2 μM) and GabD2 (0.4 μM) (squares) can convert α-ketoglutarate to succinate with the production of NADPH followed at A_{340}.

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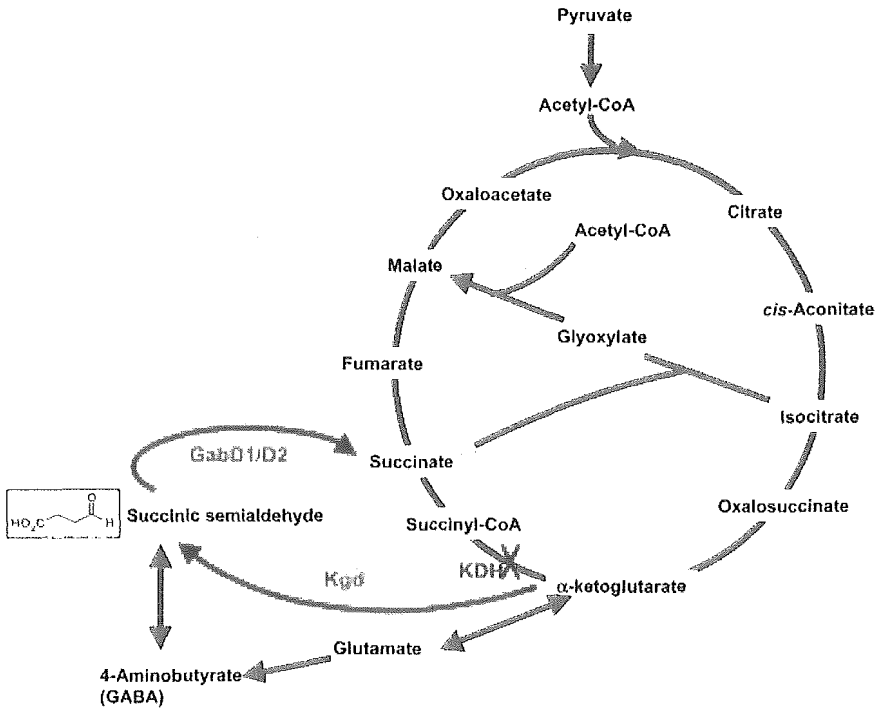

Fig. 4. TCA cycle with proposed alternative pathway for converting α-ketoglutarate to succinate in Mtb.

Discussion

Mtb and certain other mycobacteria appear to lack KDH activity (16). Thus, they might operate separate oxidative and reductive TCA half-cycles. Based on the studies presented here, we propose that Kgd along with GabD1 may form a functional link between the half-cycles, in which α-ketoglutarate is converted to succinate via two reactions that to our knowledge have not been previously observed in mycobacteria: decarboxylation of α-ketoglutarate to SSA and oxidation of SSA to succinate (Fig. 4). The proposed role for Rv1248c may explain why its disruption prevented outgrowth of the resultant mutant strain (25). Future work should attempt to disrupt *kgd* in both haploid and merodiploid backgounds to confirm its apparent essentiality.

Many TCA cycle and related enzymes in Mtb have been studied individually in recent years, such as isocitrate lyase (8), malate dehydrogenase (26), and isocitrate dehydrogenase (26, 27). However, to our knowledge, there have been no reports in which KDH activity was purified from Mtb lysates or reconstituted by using recombinant Mtb proteins. The KDH activity ascribed to Mtb lysates many years ago was $NADP^+$-dependent and CoA-independent (15). Most likely, this represented the combined actions of Kgd plus SSADH, whose coupled reactions convert α-ketoglutarate to succinate with concomitant production of NADPH in a CoA-independent manner, in contrast to the NADH-producing, CoA-dependent activity characteristic of KDH. Reports of KDH in *Mycobacterium lepraemurium* have been controversial (28–30); when seen, the activities were extremely low, and when the bacteria were studied after recovery from infected hosts, contamination by host enzymes was difficult to exclude. That *Mycobacteria* cannot utilize many organic substrates led Segal to propose, >20 years ago, that KDH may be absent, leaving a "broken" TCA cycle that subserves only biosynthetic functions (31).

Although unanticipated for Mtb, split TCA cycles with or without interconnecting pathways are common among bacteria. Out of 17 microbial genomes surveyed in ref. 30, only four appeared to encode all of the genes necessary for a complete, canonical TCA cycle. The variant cycles that occur in most species presumably reflect adaptation of their metabolism to diverse environments (32). Lack of KDH often accompanies anaerobic or microaerophilic metabolism (11).

TCA half-cycles can be joined in several ways. *Helicobacter pylori* lacks KDH, and the oxidative and reductive branches of its TCA pathway are conjoined by α-ketoglutarate ferredoxin oxidoreductase (13). We tested *M. smegmatis* and *M. bovis* bacillus Calmette–Guérin for α-ketoglutarate oxidoreductase activity, but were not able to detect it. We cannot exclude its presence, because the enzyme is labile and oxygen-sensitive (33).

To our knowledge, throughout species, only three Kgd activities have been reported: in *Euglena gracilis* (34), in *B. japonicum* (17) and in association with a bifunctional protein cloned from *E. coli*, MenD (35). The activities in *Euglena gracilis* and the *sucA* mutant strain of *B. japonicum* have not been ascribed to specific genes or proteins, so it is not possible to tell whether the enzymes involved are homologs of Mtb's Kgd. Both *Euglena gracilis* and the *sucA* mutant strain of *B. japonicum* lack KDH activities. Their Kgd activities are TPP- and Mg^{2+}-dependent but CoA-independent, as is Mtb's Kgd reaction. MenD, which is involved in biosynthesis of menaquinone (vitamin K_2) (36), catalyzes the synthesis of 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate through two reactions, the first being the decarboxylation of α-ketoglutarate (35). The product of α-ketoglutarate decarboxylation is the SSA-TPP anion rather than SSA as in Mtb. Thus, to our knowledge, Mtb's Kgd is the first enzyme of defined sequence shown to produce SSA.

SSADH activity in Mtb is encoded by both *gabD1* and *gabD2*. SSADH belongs to the aldehyde dehydrogenase family, and is well conserved in prokaryotes (37), plants (38), and mammals (24). The requirement of both GabD1 and GabD2 for $NADP^+$ is typical for bacterial SSADH, although *Pseudomonas putida* has two SSADH's, one dependent on NAD^+ and the other on $NADP^+$. Plant SSADH plays a role in antioxidant defense (39). Whether Mtb's GabD1 and GabD2 are engaged in antioxidant defense remains to be addressed.

To maintain carbon flux under oxygen-limiting conditions, bacteria that lack KDH can potentially use several bypass pathways.

The glyoxylate shunt has long been known to be operational in Mtb (7, 40). The possibility of a γ-aminobutyric acid shunt in Mtb has not been addressed. We were able to detect high levels of glutamate dehydrogenase activity in *M. smegmatis* but not in Mtb or *M. bovis* bacillus Calmette–Guérin (data not shown). In *Euglena gracilis* (34) and rhizobia such as *sucA B. japonicum*, *Mesorhizobium loti*, and *Rhizobium leguminosarum*, it was suggested that the bypass pathway from α-ketoglutarate to succinate via SSA represents an adaptation to microaerophilic conditions inside legume nodules (17). Similarly the Kgd-GabD1 bypass pathway in Mtb may help the pathogen cope with conditions in host cells that are genuinely hypoxic, or, as appears to be the case in Mtb residing in macrophages *in vitro*, misperceived as hypoxic, perhaps because Mtb's oxygen-sensing mechanisms have been damaged by host-derived nitric oxide (3). Expression of *sucA* (that is, the *kgd* homolog) has been detected in *Mycobacterium avium* during growth in human macrophages (41).

Of the metabolites whose levels were measured, the most abundant was glutamate. Whether this may be due in part to the high level of glutamate (2.6 mM) in the standard medium used to grow the bacteria is under study. To our knowledge, only one value relating to the free glutamate content of Mtb has been reported, but that value also included glutamine (42). The combined value, which was assessed enzymatically in total cell lysates, was far higher than what we detected by physical methods in the methanol extract of the soluble fraction of the cell lysate. The levels of glutamate detected in Mtb in the present work, which are estimated to correspond to 665 μmol/g dry weight (log phase) and 207 μmol/g dry weight (stationary phase), are within or above the higher values reported for *E. coli* (18), comparable to those in *Bacillus subtilis* (43) and in the lower range of values reported for *Corynebacterium glutamicum* (44). Conversion of these levels to intracellular concentrations depends on measurement of cell water content, which has apparently not been done for Mtb. For purposes of approximation, use of cell volumes estimated for other bacteria such as *E. coli* (18), *Bacillus subtilis* (43), and *Clostridium perfringens* (19) would suggest that Mtb's intracellular glutamate concentration may be on the order of 80 mM. However, Mtb's smaller size (45) and higher lipid content (see *Materials and Methods*) support estimates of intracellular glutamate concentration ranging from ≈80 mM to the low molar level. Bacteria often respond to a high osmotic environment by accumulating osmoprotectants that can be metabolized, termed "compatible solutes." Glutamate is among the small number of evolutionarily conserved compatible solutes (46) and serves as the most abundant anion in some bacteria (47). The medium we used to culture Mtb was only 175 mOsm. That Mtb nonetheless contained glutamate in what is estimated to be a high millimolar to molar range suggests that Mtb might accumulate glutamate during growth in culture as a reserve for use under more stringent conditions in the host. Accumulation of glutamate would likely exert feedback inhibition on glutamate dehydrogenase (48), and may account for our inability to detect glutamate dehydrogenase activity in Mtb and *M. bovis* bacillus Calmette–Guérin lysates.

Thus, we suggest that Mtb and some other mycobacteria may operate the TCA cycle in the half-cyclic mode characteristic of microbes adapted to low-oxygen conditions, leading to α-ketoglutarate and glutamate via the oxidative branch and succinate via the reductive branch. Both branches may be linked by Kgd and SSADH to produce succinate from α-ketoglutarate via SSA. Given that Kgd is lacking in humans, it will be of interest to identify Kgd-selective inhibitors and test their impact on the fate of Mtb during infection of macrophages.

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1. Coker, R. J. (2004) *Trop. Med. Int. Health* **9,** 25–40.
2. Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H. W., Neefs, J. M., Winkler, H., Van Gestel, J., Timmerman, P., Zhu, M., Lee, E., *et al.* (2005) *Science* **307,** 223–227.
3. Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D., Nathan, C. & Schoolnik, G. K. (2003) *J. Exp. Med.* **198,** 693–704.
4. McKinney, J. D. & Gomez, J. E. (2003) *Nat. Med.* **9,** 1356–1357.
5. Karakousis, P. C., Yoshimatsu, T., Lamichhane, G., Woolwine, S. C., Nuermberger, E. L., Grosset, J. & Bishai, W. R. (2004) *J. Exp. Med.* **200,** 647–657.
6. Shi, L., Jung, Y. J., Tyagi, S., Gemaro, M. L. & North, R. J. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 241–246.
7. Wayne, L. G. & Lin, K. Y. (1982) *Infect. Immun.* **37,** 1042–1049.
8. Honer Zu Bentrup, K., Miczak, A., Swenson, D. L. & Russell, D. G. (1999) *J. Bacteriol.* **181,** 7161–7167.
9. McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr., & Russell, D. G. (2000) *Nature* **406,** 735–738.
10. Bryk, R., Lima, C. D., Erdjument-Bromage, H., Tempst, P. & Nathan, C. (2002) *Science* **295,** 1073–1077.
11. Guest, J. R. (1995) *Philos. Trans. R. Soc. London B* **350,** 189–202.
12. Guest, H. (1987) *Biochemical Society Symposium* **54,** 3–16.
13. Pitson, S. M., Mendz, G. L., Srinivasan, S. & Hazell, S. L. (1999) *Eur. J. Biochem.* **260,** 258–267.
14. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, *et al.* (1998) *Nature* **393,** 537–544.
15. Murthy, P. S., Sirsi, M. & Ramakrishnan, T. (1962) *Biochem. J.* **84,** 263–269.
16. Tian, J., Bryk, B., Shi, S., Erdjument-Bromage, H., Tempst, P. & Nathan, C. (2005) *Mol. Microbiol.* **57,** 859–868.
17. Green, L. S., Li, Y., Emerich, D. W., Bergersen, F. J. & Day, D. A. (2000) *J. Bacteriol.* **182,** 2838–2844.
18. Mengin-Lecreulx, D., Flouret, B. & van Heijenoort, J. (1982) *J. Bacteriol.* **151,** 1109–1117.
19. Guerlava, P., Izac, V. & Tholozan, J. L. (1998) *Curr. Microbiol.* **36,** 131–135.
20. Neidhardt, F. (1987) in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, eds. Neidhardt, F., Ingraham, J., Brooks Low, K., Magasanik, B., Schaechter, M. & Edwin Umbarger, H. (Am. Soc. Microbiol. Press, Washington, DC), Vol. 1, p. 5.
21. Zinsser, H. & Joklik, W. (1988) *Zinsser Microbiology* (Appleton and Lange, Norwalk, CT).
22. Shigeoka, S. & Nakano, Y. (1991) *Arch. Biochem. Biophys.* **288,** 22–28.
23. Satya Narayan, V. & Nair, P. M. (1989) *Arch. Biochem. Biophys.* **275,** 469–477.
24. Chambliss, K. L., Caudle, D. L., Hinson, D. D., Moomaw, C. R., Slaughter, C. A., Jakobs, C. & Gibson, K. M. (1995) *J. Biol. Chem.* **270,** 461–467.
25. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. (2003) *Mol. Microbiol.* **48,** 77–84.
26. Ohman, R. & Ridell, M. (1996) *Tuber Lung Dis.* **77,** 454–461.
27. Florio, W., Bottai, D., Batoni, G., Esin, S., Pardini, M., Maisetta, G. & Campa, M. (2002) *Clin. Diagn. Lab. Immunol.* **9,** 846–851.
28. Mori, T., Kosaka, K. & Tanaka, Y. (1971) *Int. J. Lepr. Other Mycobact. Dis.* **39,** 796–812.
29. Ishaque, M., Togola, D. & Sticht-Groh, V. (1994) *Int. J. Lepr. Other Mycobact. Dis.* **62,** 399–403.
30. Cordwell, S. J. (1999) *Arch. Microbiol.* **172,** 269–279.
31. Segal, W. (1984) *Mycobacteria: A Sourcebook*, eds. Kubica, G. P. & Wayne, L. G. (Dekker, New York), Part A, pp. 547–573.
32. Huynen, M. A., Dandekar, T. & Bork, P. (1999) *Trends Microbiol.* **7,** 281–291.
33. Mai, X. & Adams, M. W. (1996) *J. Bacteriol.* **178,** 5890–5896.
34. Shigeoka, S., Onishi, T., Maeda, K., Nakano, Y. & Kitaoka, S. (1986) *FEBS Lett.* **195,** 43–47.
35. Palaniappan, C., Sharma, V., Hudspeth, M. E. & Meganathan, R. (1992) *J. Bacteriol.* **174,** 8111–8118.
36. Bentley, R. & Meganathan, R. (1982) *Microbiol. Rev.* **46,** 241–280.
37. Sanchez, M., Alvarez, M. A., Balana, R. & Garrido-Pertierra, A. (1988) *Biochim. Biophys. Acta* **953,** 249–257.
38. Busch, K., Piehler, J. & Fromm, H. (2000) *Biochemistry* **39,** 10110–10117.
39. Bouche, N., Fait, A., Bouchez, D., Moller, S. G. & Fromm, H. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 6843–6848.
40. Kannan, K. B., Katoch, V. M., Bharadwaj, V. P., Sharma, V. D., Datta, A. K. & Shivannavar, C. T. (1985) *Indian J. Lepr.* **57,** 542–548.
41. Hou, J. Y., Graham, J. E. & Clark-Curtiss, J. E. (2002) *Infect. Immun.* **70,** 3714–3726.
42. Cowley, S., Ko, M., Pick, N., Chow, R., Downing, K. J., Gordhan, B. G., Betts, J. C., Mizrahi, V., Smith, D. A., Stokes, R. W. & Av-Gay, Y. (2004) *Mol. Microbiol.* **52,** 1691–1702.
43. Markuszewski, M. J., Otsuka, K., Terabe, S., Matsuda, K. & Nishioka, T. (2003) *J. Chromatogr. A* **1010,** 113–121.
44. Uy, D., Delaunay, S., Goergen, J. L. & Engasser, J. M. (2004) *Bioprocess Biosyst. Eng.* **27,** 153–162.
45. Bergey, D. H., Holt, J. & Krieg, N. (1993) *Bergey's Manual of Determinative Bacteriology* (Williams and Wilkins, Baltimore).
46. Kempf, B. & Bremer, E. (1998) *Arch. Microbiol.* **170,** 319–330.
47. Csonka, L. N. (1989) *Microbiol. Rev.* **53,** 121–147.
48. Shiio, I. & Ozaki, H. (1970) *J. Biochem. (Tokyo)* **68,** 633–647.