

Complement-mediated hemolysis: The involvement of oxidative stress and the ameliorating effect of Fermented Papaya Preparation

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Abstract

Objectives We studied the involvement of serum-complement (C') and oxidative stress in the hemolytic anemias Paroxysmal Nocturnal Hemoglobinuria (PNH) and Auto-Immune Hemolytic Anemia (AIHA), and the effect of Fermented Papaya Preparation (FPP), an antioxidant-containing yeast fermentation product of *Carica papaya* Linn.

Methodology Normal human red blood cells (RBC) treated with the sulphhydryl compound 2-aminoethyl isothiuronium bromide demonstrated a PNH-phenotype (reduced surface expression of the protecting CD55 and CD59 antigens). AIHA was simulated by treating group-A RBC with anti-A antibodies. Both samples were exposed to C'-containing, O-type, serum.

Results Hemolysis, which was developed 40 min later, was preceded by an abrupt increase in reactive oxygen species (ROS). Both ROS and hemolysis were reduced by inactivating the C' (by heat or by an anti-C' antibody), and by FPP (20 mg/ml), indicating the involvement of C' and oxidative stress, respectively. One PNH patient treated with FPP showed reduced hemolysis.

Conclusion Currently, a humanized anti-C' monoclonal antibody is the main treatment for PNH and some other hemolytic anemias. Our results suggest a therapeutic role for antioxidants. Since FPP is well tolerated and relatively inexpensive, its use may be considered as an alternative or adjuvant therapy for PNH and other C'-mediated hemolytic anemias.

List of abbreviations: Complement (C'), Paroxysmal Nocturnal Hemoglobinuria (PNH), phosphatidylinositol glycan complementation class A (*PIG-A*), Auto-Immune Hemolytic Anemia (AIHA), Fermented Papaya Preparation (FPP), red blood cells (RBC), reactive oxygen species (ROS), glycosylphosphatidylinositol (GPI), mean fluorescence channel (MFC), Phosphatidylserine (PS).

INTRODUCTION

The complement (C') and the redox systems play important roles in the physiological functioning of the body, such as in the defense system, but they are also involved in various pathological conditions, including hemolytic anemia. Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare clonal disorder caused by acquired somatic mutations in the phosphatidylinositol glycan complementation class A (*PIG-A*) gene. This gene encodes for an enzyme in the production pathway of the glycosylphosphatidylinositol (GPI) anchor, by which various proteins are

attached to the cell membrane. A mutation in this gene in a hematopoietic stem cell causes a deficiency in GPI-anchored proteins, including CD55 and CD59 that protect the RBC against C'-mediated destruction. Autoimmune hemolytic anemia (AIHA) is caused by auto-antibodies that bind to surface RBC antigens. In both diseases, RBC are sensitive to C'-mediated destruction, resulting in intra- and extra-vascular hemolysis.

Antioxidants ameliorate oxidative stress by preventing generation of reactive oxygen species (ROS), by

scavenging and preventing their accumulation and by correcting their cellular damage.

Using experimental model systems, we studied the interaction between the C' and the redox systems in C'-mediated hemolysis of PNH and AIHA, and the ameliorating effect of Fermented Papaya Preparation (FPP), an antioxidant-containing product of yeast fermentation of *Carica papaya* Linn.

METHODS

RBC were obtained from normal and PNH donors. PNH-like RBC were generated by treating normal human RBC with the sulphhydryl compound 2-aminoethyl isothiuronium bromide (Fibach and Dana, 2015). The PNH-like phenotype was manifested by reduced surface expression of CD55 and CD59 (not shown). AIHA was simulated by treating group-A RBC with anti-blood group-A antibodies. Both treated RBC were incubated with fresh (C'-containing) autologous or O-type serum. Reactive oxygen species (ROS), as a parameter of oxidative stress, were measured by flow cytometry and the mean fluorescence channel (MFC) determined (Amer *et al.*, 2008). Hemolysis was determined after 20 min by spectro-photometric measurement of hemoglobin in the supernatant (Fibach and Dana, 2015) and presented as percent compared to RBC completely hemolyzed in water). FPP was dispersed in saline.

RESULTS

The results (Fig. 1) show that the ROS and hemolysis were increased in PNH, PNH-like and AIHA-like RBC compared to normal RBC, and were further increased following exposure to C'. Inactivation of the C', either by heat (56°C for 30 min) (Fig. 1C) or by the addition of an anti-C' antibody (not shown), effectively reduced the ROS generation and hemolysis, indicating that both are mediated by active C'. FPP, added during the exposure to activated C' had a dose-related reducing effect on ROS generation and hemolysis (see Fig. 1 B,C. for the optimal concentration (20 mg/ml),

CONCLUSIONS

The potential sites of involvement of ROS in C'-mediated hemolysis in PNH and AIHA are schematically presented in Fig. 2 (for details see Fibach and Dana, 2015).

Currently, a humanized anti-C' monoclonal antibody is the main treatment for PNH and some other hemolytic anemias. Our results suggest a therapeutic role for antioxidants. Since FPP is well tolerated and relatively inexpensive, its use may be considered as an alternative or adjuvant therapy for PNH and other C'-mediated hemolytic anemias. Its optimal route of administration (per os or by injection) requires further study.

ACKNOWLEDGEMENT

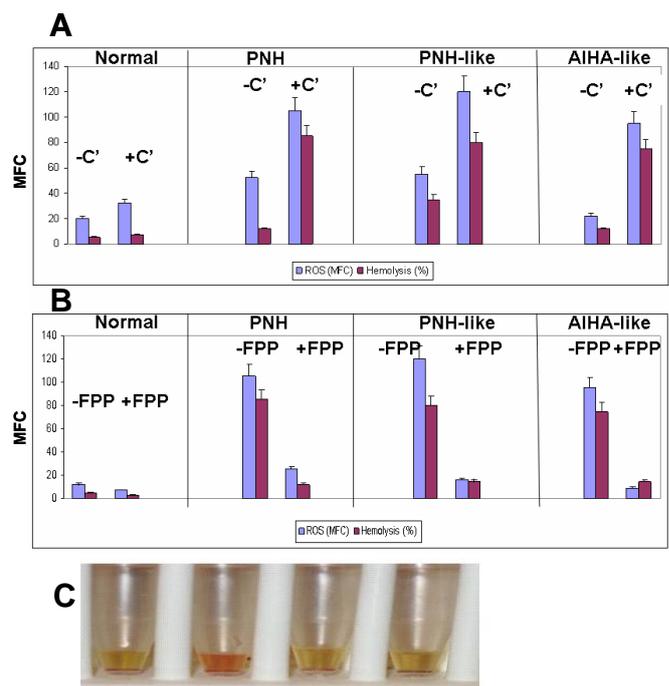
We thank the Osato Research Institute (Gifu, Japan) for their financial support.

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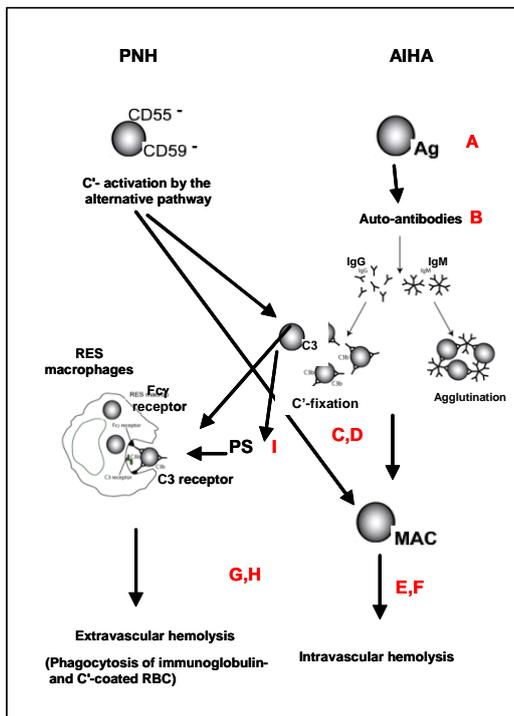
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Figure 1. Reactive oxygen species (ROS) and hemolysis in PNH and AIHA-like RBC and the effect of complement (C') and Fermented Papaya Preparation (FPP).



RBC were obtained from normal and PNH donors; PNH- and AIHA-like RBC were generated, and ROS and hemolysis were measured as detailed in Methods. A. RBC were incubated with fresh (C'-containing, +C') autologous or heated 56°C for 30 min (C'-inactivated, -C') plasma. B. RBC were incubated with fresh (C'-containing) autologous plasma with or without 20 µg/ml FPP. C. Hemolysis in PNH-like RBC. RBC were untreated (a), treated with C' (b), treated with inactivated C' (c), treated with C' and FPP (d). The results show that the ROS and hemolysis were increased in PNH, PNH-like and AIHA-like RBC compared to normal RBC, and were further increased following exposure to C'. FPP significantly ameliorated the increased ROS and hemolysis.

Figure 2. The potential involvement of reactive oxygen species (ROS) in complement-mediated hemolysis in PNH and AIHA



A schematic presentation of the potential sites (marked by alphabet letters) of oxidative stress involvement, through generation of unbalanced ROS, in immune and non-immune hemolytic anemias. (A) Enhancement of senescent signal expression. (B) Stimulation of auto-antibody production. (C) Induction of ROS by C³-fixation. (D) Facilitation of C³-fixation by ROS. In intravascular hemolysis, ROS may serve as the proximal membrane perforation agent as part of (E) or in parallel to (F) the membrane attack complex (MAC). Following hemolysis, the leaked RBC content may contain (G) oxidants (heme, hemoglobin, hemichromes and free iron) which induce ROS generation as well as (H) antioxidants (superoxide dismutase, catalase) with an opposite effect. (G) In extravascular hemolysis, (I) ROS may induce eryptosis, including externalization of phosphatidylserine (PS), whereby damaged cells are phagocytosed by macrophages. Modified from: Fibach and Dana, 2015.