IS *PENICILLIUM CITRINUM* IMPLICATED IN SAGO HEMOLYTIC DISEASE?

Latifeh Atagazli[1], Andrew R Greenhill[1,2], Wayne Melrose[3], Aisak G Pue[4] and Jeffrey M Warner[1]

[1]Environmental and Public Health Research Group, Microbiology and Immunology, James Cook University, Townsville, Queensland, Australia; [2]Infection and Immunity, Institute of Medical Research, Goroka, Papua New Guinea; [3]School of Public Health and Tropical Medicine, James Cook University, Townsville, Queensland, Australia; [4]The Animal Research Institute, Biosecurity Queensland, Department of Employment, Economic Development and Innovation, Yeerongpilly, Queensland, Australia

Abstract. Sago hemolytic disease (SHD) is an acute hemolytic syndrome affecting rural Papua New Guineans who depend on the starch of *Metroxylon sagu* as a staple carbohydrate. It is a suspected mycotoxicosis associated with fungal succession in stored and perhaps poorly fermented sago. Despite a mortality rate of approximately 25%, little is know about the disease. Recent studies have identified *Penicillium citrinum* as a possible candidate in the etiology of SHD. This is based on the frequency of isolation from sago starch and the hemolytic nature of the organism as demonstrated when cultured on sheep and human blood agar. A highly non-polar lipophilic *P. citrinum* fraction from C18 solid phase extraction demonstrated high hemolytic activity in a semi-quantitative assay using both mouse and human erythrocytes. When the red cell membrane proteins were subjected to sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, cleavage of protein band 3 and spectrin was demonstrated. This breach of major structural red cell proteins is consistent with the severe hemolysis found *in vivo*. Our findings warrant further investigation into the hemolytic activity of *P. citrinum* and its role as the etiological agent of SHD.

Key words: *Penicillium citrinum*, sago hemolytic disease, mycotoxicosis

INTRODUCTION

Subsistence dwellers in lowland areas of Papua New Guinea (PNG) derive the majority of their dietary carbohydrates from the pith of the sago palm, *Metroxylon sagu*. Familial outbreaks of acute intravascular hemolysis, known as sago hemolytic disease (SHD), have been associated with the consumption of stale sago starch (Taufa, 1974; Donovan *et al*, 1976, 1977). The disease is characterized by acute hemolytic anemia, severe dehydration, fever, headache, vomiting, jaundice, lethargy, grinding of teeth, hematuria and splenomegaly. Clinical signs of SHD develop rapidly and are associated with a mortality rate of approximately 25%, with

Correspondence: Jeffrey Warner, Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, QLD, 4811, Australia.
Tel: +61 7 4781 6375; Fax: +61 7 4779 1526
E-mail: Jeffrey.Warner@jcu.edu.au

death typically occurring within 72 hours of disease onset (Taufa, 1974; Donovan *et al*, 1976, 1977). The incidence of SHD remains speculative, but is likely to be under diagnosed because of a lack of healthcare facilities where it is endemic (Greenhill, 2006).

On account of the apparent foodborne nature of the illness and the rapid onset of symptoms, it seems likely microbial contamination, and more specifically, preformed microbial toxins, play a role in SHD. Although several studies of the microbiology of sago starch have been conducted (Donovan *et al*, 1977; Greenhill *et al*, 2007a, b) the etiology of SHD remains elusive. The absence of common foodborne mycotoxins in sago starch (Greenhill *et al*, 2008) led to studies that identified a number of organisms that were intensely hemolytic *in vitro* (Greenhill *et al*, 2010a). One of the most commonly occurring organisms was *Penicillium citrinum*, which was isolated from both stale sago and sago considered fit for consumption (Greenhill, 2006). The purpose of this study was to investigate the *in vitro* hemolytic activity of a fraction of *P. citrinum* and to determine its action on human erythrocyte proteins. We also compared the activity of the fraction in human and mouse erythrocytes, to gauge the viability of using mice as an animal model for SHD research.

MATERIALS AND METHODS

Fungal extracts

P. citrinum was cultured on moist sterile wheat and extractions conducted in hexane, as previously described (Greenhill *et al*, 2010a). Extractions were fractionated using methanol:water (80:20) on a C18 solid phase extraction column (Varian Bond Elut LRC-SI, Australia). Activity driven fractionation using the hemolytic assay developed by Greenhill *et al* (2010a) resulted in a non-polar lipophilic hemolytic fraction that was dried and stored at -20ºC. The extract was resuspended in methanol at a concentration of 1 g/ml as a stock solution, from which working concentrations were made.

Human and mouse erythrocytes

Blood samples were taken from a human volunteer (n=1) and male 7-13 week old BALB/c mice (n=15). Mice were euthanized with CO_2 and blood was subsequently collected from the aorta. The use of the animals was approved by the Ethics Committee for the Use of Animals at James Cook University.

Hemolytic assay

Screening for hemolytic activity was based on the method developed by Greenhill *et al* (2010a) with minor modifications to adjust for the fragility of mouse blood. Whole blood was centrifuged at room temperature in a microcentrifuge for 5 minutes at appropriate centrifugation speeds to ensure the physical integrity of erythrocytes was maintained (3,000*g* for human blood, 1,000*g* for mouse blood). The plasma and buffy coat were removed and erythrocytes were washed in 20 volumes of phosphate buffered saline (PBS) and centrifuged as above. This process was repeated three times.

Slightly different volumes of human and mouse erythrocytes were used so a comparable mass of erythrocytes was used in both assays, taking into account the different sizes of human and mouse erythrocytes. Dilutions were made in PBS until the packed cell volume was comparable for both species.

For quality control, the hemolytic activity of *P. citrinum* was compared to *C. perfringens* phospholipase C (Sigma, NY), as its hemolytic activity has previously

been established (Bowman *et al*, 1971; Sakurai *et al*, 2004).

Human and mouse erythrocyte suspensions (150 μl) were incubated with 0.5 mg of *P. citrinum* fraction or phospholipase C. Both the *P. citrinum* fraction and phospholipase C were at a final concentration of 3.3 μg/μl. Duplicate negative controls (reagent blank) and one positive control (erythrocytes completely hemolysed by the addition of 50 μl of 10% Triton-X 100) were used. Sampling occurred after incubation periods of 30, 60, 120 and 240 minutes. The samples were centrifuged (1,000*g*, room temperature, 5 minutes) and absorbance of the supernatant was read at 540 nm (Labsystems Multiscan EX, Pathtech, Australia). The negative control values obtained in the assay were subtracted from the test values and presented as percentage hemolysis of the positive control.

Extract activity on erythrocyte membrane proteins

Equal concentrations of hemoglobin-free human and mouse erythrocyte membranes (ghost cells) were prepared as described by Dodge *et al* (1963). This was to accurately compare the proteolytic activity of the *P. citrinum* fraction on the two species. Protein estimation was conducted on the ghost cells using the BCA Protein Assay Kit (Pierce, IL), following the manufacturer's instructions.

Aliquots of the hemolytic fraction of *P. citrinum* (12.5 μg/μl) were added to an equal volume of erythrocyte proteins (750 μg/ml) to give a final concentration of 6.25 μg/μl in a 30 μl reaction mixture. Reactions were incubated at 37ºC for 1 hour. Two sets of negative controls were employed; ghost cells in lysis buffer, and the *P. citrinum* fraction in lysis buffer (12.5 μg/μl). The positive control consisted of phospholipase C in lysis buffer (2.5 μg/μl) and 750 μg/ml erythrocytes. All reactions were incubated at 37ºC for 1 hour.

Following incubation, 15 μl of each sample was added to 15 μl of sample buffer and heat treated at 100ºC for 3 minutes in preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein separation of samples was conducted using 10% polyacrylamide gel (Pierce, Australia). The gel was run in a Mini-PROTEAN 3 SDS-PAGE tank (Bio-Rad, CA) and electrophoresed at 100 V for 60 minutes in Tris-HEPES-SDS running buffer (Pierce, IL). Thereafter, the gel was stained with silver stain. Images were captured by digital photography with Gel Doc 1000 hardware (BioRad, CA) and a hand held digital camera.

RESULTS

Hemolytic activity

The hemolytic assay demonstrated the non-polar lipophilic fraction from *P. citrinum* is hemolytic *in vitro*. There was a notable difference observed in the hemolytic activity using mouse and human erythrocytes. In human erythrocytes the *P. citrinum* fraction and phospholipase C were both highly active, resulting in 80-100% hemolysis within 30 minutes. In comparison, 50-80% hemolysis was observed in mouse erythrocytes (Fig 1).

Activity of fraction on erythrocyte membrane proteins

Qualitative differences were observed by SDS-PAGE between species. Proteolytic activity of the *P. citrinum* fraction was detected on both human and mouse erythrocyte membrane proteins (Figs 2 and 3). The *P. citrinum* fraction incubated with the human membrane proteins resulted in changes to α and β spectrin, ankryin and in particular protein band 3, relative to the

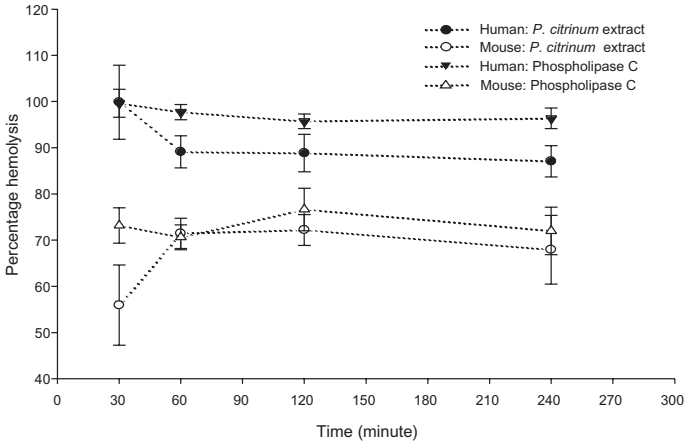

Fig 1–Hemolytic activity of *Penicillium citrinum* fraction (3.3 μg/μl) and phospholipase C toxin (3.3 μg/μl) on human and mouse erythrocytes.

control band. No extra bands could be detected as a result of this proteolytic activity. The phospholipase C positive control was also observed to have proteolytic activity on human erythrocyte membrane proteins spectrin and protein band 3 (data not shown). Similarly, for mouse erythrocyte membrane proteins the *P. citrinum* fraction was proteolytic on spectrin and protein band 3. A low molecular weight protein (approximately 58 kDa) and the appearance of another band (approximately 32 kDa) were observed on samples incubated with the *P. citrinum* fraction. The positive control, phospholipase C, had proteolytic activity similar to the *P. citrinum* fraction, acting mainly on α and β spectrin and protein band 3 without the appearance of extra bands on the SDS-PAGE (data not shown).

DISCUSSION

We have demonstrated the hemolytic activity of the *P. citrinum* fraction used in the current study is proteolytic and acts on major structural proteins of the red cell membrane. The fraction was active on human erythrocytes, consistent with a role in human disease; and mouse erythrocytes, suggesting that an animal model may be useful in future studies to further elucidate the pathophysiology of this disease.

Two different forms of cleavage were observed by the *P. citrinum* fraction on human and mouse erythrocyte membrane protein band 3 (Figs 2 and 3). On silver stained human erythrocyte membrane protein, protein band 3 was less diffuse than the control (Fig 2). No extra bands could be identified with the highly sensitive silver stained gel. However, in mouse erythrocyte membrane proteins treated with the *P. citrinum* fraction, two additional bands could be observed of approximately 58 and 35 kDa (Fig 3). The estimated molecular weight of these two bands add up to 93 kDa, which closely approximates the molecular weight of the erythrocyte membrane protein band 3. This suggests the proteolytic activity of the *P. citrinum* extract is predominantly toward protein band 3. Although band A (α and β spectrin) also appeared to be affected by *P. citrinum* extract, the size of the new protein bands probably does not represent spectrin, a larger molecule with a molecular weight of approximately 240 kDa. The slight differences seen between the hemolytic activity of the *P. citrinum* fraction on human and mouse erythrocytes in the hemolytic assay and the nature of the proteolytic activity observed in this study may be due to slight structural differences between the mouse and human protein band 3. Glycophorins on the mouse erythrocyte exist in lower concentrations than

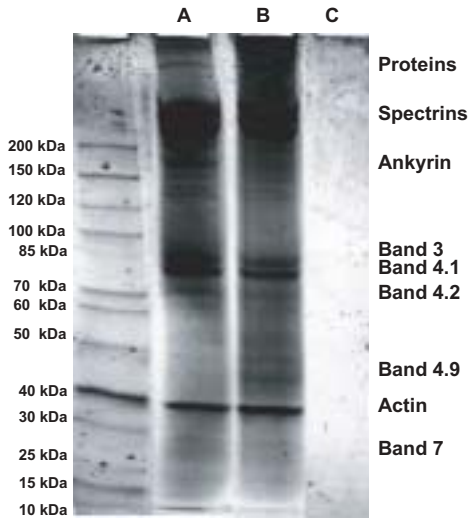

Fig 2–SDS-PAGE pattern of human erythrocyte membrane proteins in 10% polyacrylamide gel following silver-staining. Lanes from left to right are: (A) negative control consisting only of erythrocyte membrane proteins, (B) erythrocyte membrane protein incubated with 6.25 μg/μl of *P. citrinum*, and (C) negative control consisting of *P. citrinum* extract only. All reactions were incubated for 1 hour at 37ºC.

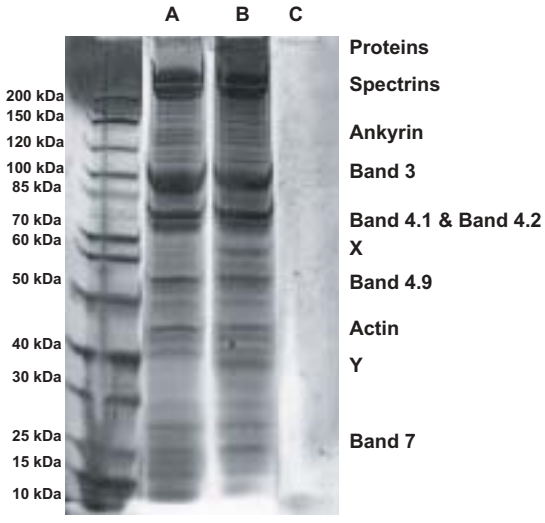

Fig 3–SDS-PAGE pattern of mouse erythrocyte membrane proteins in 10% polyacrylamide gel, following silver-staining. Note that the letters X and Y indicate the identification of new protein bands. Lanes from left to right are: (A) negative control consisting only of erythrocyte membrane proteins, (B) erythrocyte membrane protein incubated with 6.25 μg/μl of *P. citrinum* extract, and (C) negative control consisting of *P. citrinum* extract only. All reactions were incubated for 1 hour at 37ºC.

in humans and structural differences also exist between the erythrocytes of the two species (Howard *et al*, 1979).

Many of the hemolytic compounds isolated from bacteria and fungi are proteins, peptides or lipophilic compounds (Schmitt *et al*, 1999; Bennett and Klich, 2003). Phospholipase C produced by bacteria such as *C. perfringens* is a well characterized toxin capable of causing hemolysis both *in vitro* and *in vivo* (Matsumoto, 1961). Phospholipase C hydrolyses membrane phospholipids in humans which results in hemolysis secondary to osmotic pressure which bursts the cell (Bowman *et al*, 1971). In this study, similar *in vitro* hemolytic activity of the *P. citrinum* fraction and phospholipase C was observed in mice and human erythrocytes.

The occurrence of SHD negatively impacts food safety and food security in areas of high sago dependency. Although efforts have been made to address the food safety of sago starch (Greenhill *et al*, 2010b), there will remain a risk of disease until the etiology is determined. This study has made important preliminary findings and paves the way for further investigations to elucidate the role of *P. citrinum* in the etiology of SHD, which are ongoing.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Centre for International Agricultural Research. We thank Lisa Elliot for technical assistance setting up the SDS-PAGE analysis.

REFERENCES

Bennett JW, Klich M. Mycotoxins. *Clin Microbiol Rev* 2003; 16: 497-516.

Bowman MH, Ottolenghi AC, Mengel CE. Effects of phospholipase C on human erythrocytes. *J Membr Biol* 1971; 4: 156-64.

Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys* 1963; 100: 119-30.

Donovan K, Shaw DE, Amato D. Sago haemolysis: clinical features and microbiological studies. *PNG Med J* 1977; 20: 167-74.

Donovan KO, Shaw DE, Amato D. Sago and haemolysis. *PNG Med J* 1976; 19: 183-4.

Greenhill AR. Food safety and security of sago starch in rural Papua New Guinea. Townsville: James Cook University, 2006.

Greenhill AR, Blaney BJ, Shipton WA, Frisvad J, Pue A, Warner JM. A survey for mycotoxins and toxigenic fungi in sago starch from Papua New Guinea. *Lett Appl Microbiol* 2008; 47: 342-7.

Greenhill AR, Blaney BJ, Shipton WA, Pue A, Fletcher MT, Warner JM. Haemolytic fungi isolated from sago starch in Papua New Guinea. *Mycopathologia* 2010; 169: 107-15.

Greenhill AR, Shipton WA, Blaney BJ, *et al*. Hazards and critical control points for traditional sago starch production in Papua New Guinea: Implications for food safety education. *Food Control* 2010b; 21: 657-62.

Greenhill AR, Shipton WA, Blaney BJ, Warner JM. Fungal colonization of sago starch in Papua New Guinea. *Int J Food Microbiol* 2007a; 119: 284-90.

Greenhill AR, Shipton WA, Omoloso AD, Amoa B, Warner JM. The prevalence of bacteria of public health significance in sago starch in Papua New Guinea. *J Food Prot* 2007b; 70: 2868-72.

Howard RJ, Smith PM, Mitchell GF. Identification of differences between the surface proteins and glycoproteins of normal mouse (Balb/c) and human erythocytes. *J Membr Biol* 1979; 49: 171-98.

Matsumoto M. Studies on phospholipids: II. Phospholipase activity of *Clostridium perfringens* toxin. *J Biochem* 1961; 49: 23-31.

Sakurai J, Nagahama M, Oda M. *Clostridium perfringens* alpha-toxin: characterization and mode of action. *J Biochem (Tokyo)* 2004; 136: 569-74.

Schmitt CK, Meysick KC, O'Brien AD. Bacterial toxins: friends or foes? *Emerg Infect Dis* 1999; 5: 224-34.

Taufa T. Sago haemolytic disease. *PNG Med J* 1974; 17: 227-8.