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POSSIBLE INVOLVEMENT OF RED CELL MEMBRANE PROTEINS IN THE HEMOLYTIC ACTION OF PORTUGUESE MAN-OF-WAR TOXIN[1]

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SUMMARY

Glycophorin and the fragments isolated from trypsinizing intact rat, dog, sheep and human red blood cells (rbc's) neutralize the hemolytic action of the Portuguese Man-of-War venom. This action can be blocked by rabbit antisheep hemolysin and phytohemagglutinin, a lectin which preferentially binds to glycophorin. Concanavalin A, which binds to band-3 protein of rbc membranes, does not block the neutralizing action of rbc tryptic fragments or glycophorin. The concentrations of rat, dog, human and sheep glycophorin which half neutralize venom induced hemolysis are inversely and linearly proportional to the hemolytic sensitivities of these rbc's to the venom. These data implicate glycophorin as a possible binding site for the hemolytic component of the Portuguese Man-of-War venom.

INTRODUCTION

Portuguese Man-of-War (Physalia physalis; PMOW) venom possesses a potent and labile hemolytic glycoprotein (MW = 250,000) (1). We view the hemolytic mechanism as involving at least two steps: an initial binding of the hemolytic component to sites on the red blood cell (rbc), and a subsequent lesion of the membrane to cause lysis. To elucidate the initial step of the hemolytic mechanism we have attempted to identify and isolate components of the rbc membranes which can neutralize the hemolytic action of PMOW venom. In this paper we have shown that low concentrations of glycophorins and band-3 proteins isolated from the rbc membranes of various mammalian species can neutralize the hemolytic action of PMOW venom. Only, the neu-

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tralizing effect of glycophorin, however, is linearly related to the sensitivity of these rbc's to venom induced hemolysis. An abstract of this work has been presented elsewhere (2).

MATERIALS AND METHODS

DIDS (4,4'-diisothiocyano-2,2'-dihydrostilbene disulfonic acid) was obtained from Pierce Chemical Co., Rockford, Ill. Lectins and rabbit antisheep hemolysin were supplied by Grand Island Biological Co., Grand Island, New York. Porcine pancreatic trypsin (Type IX, crystalline) and soybean trypsin inhibitor were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade. Lithium diiodosalicylate (LIS) was prepared from recrystallized 3,5-diiodosalicylic acid, and lithium hydroxide as described by Marchesi and Andrew (3).

PMOW nematocyst venom was prepared as described (1). A 12.5% (v/v) suspension of rbc's in isotonic Tris-saline (10 mM Tris-Cl, pH 7.4 and 144 mM NaCl) was prepared (4) fresh daily and used as a stock for hemolytic assays. Unless otherwise stated, the venom's hemolytic activity was assayed in duplicate with 4 ml of a 0.625% (v/v) suspension of rbc's in Tris-saline. Ten µl of PMOW venom containing 0.03 to 0.07 µg of protein was added to each rbc suspension at 0^{o}. The mixtures were incubated at 30^{o} for 30 min with constant shaking. Following centrifugation at 1,500 x g for 5 min, the A_{570} (absorbancy at 570 nm) of the supernatant was measured and expressed as a percent lysis based on supernatant from a 0.625% suspension of rbc's lysed in water. When testing glycophorin or other substances for their ability to neutralize the hemolytic activity of PMOW venom they were premixed with the venom in a volume of 0.1 ml before the addition of rbc's.

Trypsinization and DIDS treatment were performed as described by Winzler et al (5) and Cabantchik and Rothestein (6), respectively. Tryptic fragments from intact rbc's and glycophorins were isolated by the methods of Winzler et al. (5) and Marchesi and Andrews (3), respectively, with the following modifications. Lyophilized tryptic-fragment or glycophorin preparations were extracted twice with absolute ethanol and three times with chloroform/methanol/acetic acid (6/3/2, v/v) at room temperature in order to remove residual contaminating phospholipids as detected by the method of Ames (7). The preparations were then dialyzed against Tris-saline overnight at 4^{o}, and cleared by centrifugation at 27,000 x g for 20 min. Band-3 proteins were prepared by the procedure of Findlay (8). Protein was determined by the method of Lowry et al. (9) using bovine serum albumin as a standard.

RESULTS

Sensitivity of rbc's to PMOW venom

Rbc's from four species of mammals are lysed to different extents by the same amount of PMOW venom (Table 1). The order of sensitivity to venom-induced hemolysis, ranging from highest to lowest, was rat, dog, human and sheep. Trypsinization of rat rbc's caused the extent of hemolysis to decrease from 33.7% to 1.7%, while that of human rbc's was increased from 10.7% to 27.5%. Trypsinization had little effect on the responses of dog

Table 1. Venom-induced hemolysis of trypsin-treated and untreated mammalian rbc's[a]

Species	Percent Hemolysis	
	Untreated	Trypsin Treated
Rat	33.7	1.7
Dog	23.9	25.6
Human	10.7	27.5
Sheep	5.6	5.0

[a]The concentration of PMOW venom used is 0.04 μg protein/ml. See Materials and Methods for details.

and sheep rbc's. Treatment of the various rbc's with soybean trypsin inhibitor alone did not significantly affect their hemolytic sensitivity to PMOW venom.

Effect of rbc tryptic fragments on PMOW lytic activity

Tryptic fragments isolated from intact rat, dog and sheep rbc's blocked the hemolytic action of PMOW venom on intact rat rbc's (Fig. 1). The concentrations of rat, dog and sheep tryptic fragments required to half neutralize (I_{50}) the hemolytic activity of PMOW venom on rat rbc's are 0.14, 0.20 and 1.75 mg/ml, respectively. Tryptic fragments from human rbc's have no effect on venom-induced hemolysis of rat rbc's.

Effect of glycophorin and band-3 protein on PMOW lytic activity

Since in the case of human rbc's the tryptic fragments are derived from the membrane protein, glycophorin (10, 11), we determined the effect of added glycophorin on the hemolytic action of PMOW venom. Glycophorins isolated from rat, dog, sheep and human rbc's all neutralize the hemolytic activity of PMOW venom in a dose dependent manner (Fig. 2). The glycophorins isolated from previously trypsinized sheep and human rbc's are much more effective at neutralizing hemolytic activity than their respective glycophorins derived from untreated rbc's. The I_{50} values of rat, dog,

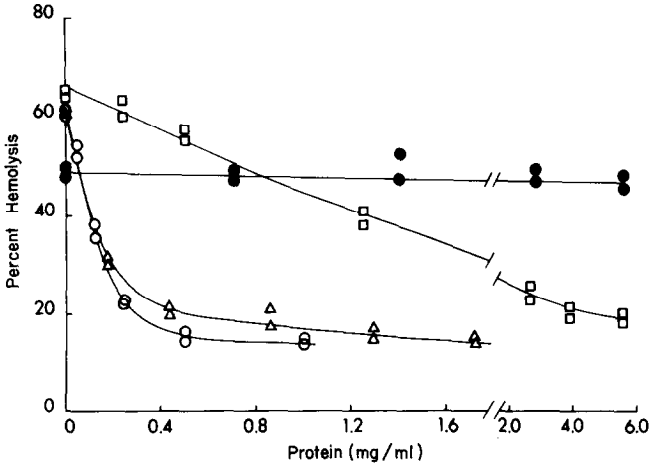

Fig. 1. Ability of rbc tryptic fragments to neutralize the hemolytic activity of PMOW venom. Different concentrations of tryptic fragments isolated from rat (o), dog (Δ), sheep (□) and human (●) rbc's were mixed with PMOW venom (final concentration of 0.7 μg/ml) at 0°. Five min later washed rat rbc's were added to each mixture to a final volume of 4 ml and a concentration of 0.625% (v/v). The extents of hemolysis were then determined.

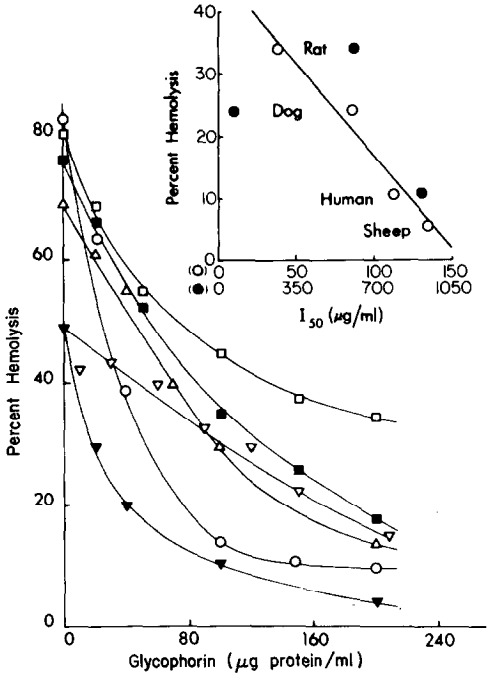

Fig. 2 Ability of glycophorin to neutralize the hemolytic activity of PMOW venom. Glycophorins were isolated from rat (o), dog (Δ), sheep (□) and human (▽) rbc's and from the trypsinized sheep (■)

human and sheep glycophorins are 38, 86, 112 and 134 μg/ml, respectively. These I_{50} values are inversely and linearly related to the sensitivity of the respective rbc's to the hemolytic action of PMOW venom (Inset of Fig. 2).

Band-3 proteins isolated from rat, dog and human rbc's were also able to neutralize the hemolytic action of PMOW venom and yield I_{50} values of 600, 60 and 910 μg/ml, respectively. The band-3 protein of sheep rbc's, in concentrations up to 153 μg/ml, had no such effect. There was, however, no linear correlation between the I_{50} values for the band-3 proteins and the hemolytic sensitivity of the respective rbc's to the venom. Such non-rbc proteins as bovine serum albumin, yeast glucose 6-phosphate dehydrogenase and beef-heart cytochrome C at levels up to 2 mg/ml, or phytohemagglutinin (PHA) at levels up to 1 mg/ml had no detectable neutralizing effects on the venom. Prior treatment of rat rbc's with the lectin did not alter their sensitivity to the venom.

Ability of specific antisera and lectins to block neutralizing action of rbc membrane components was tested. Tryptic fragments or glycophorins were mixed with the rabbit antisheep hemolysin or rabbit nonimmune serum prior to the addition of PMOW venom. The neutralizing activities of sheep tryptic fragments and glycophorin are partially blocked by pretreating with the hemolysin while preimmune serum showed little effect (Fig. 3). Smaller decreases in the neutralizing activities of rat and human glycophorins also occurred. The ability of the hemolysin to block the neutralizing activities of these membrane components is dose dependent. The hemolysin alone has no significant effect on the venom-induced hemolysis. The neutralizing effect of rat band-3 protein on the venom's hemolytic activity is not blocked by the antiserum.

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and human (▼) rbc's. The procedure for determining their inhibitory effect on venom-induced hemolysis of rat rbc's is the same as in Fig. 1.

Inset: Relationships of the I_{50} values for glycophorins (o) from rbc's of various species to the hemolytic susceptibility of these cells to PMOW venom. The I_{50} values for band-3 proteins (●) are also shown.

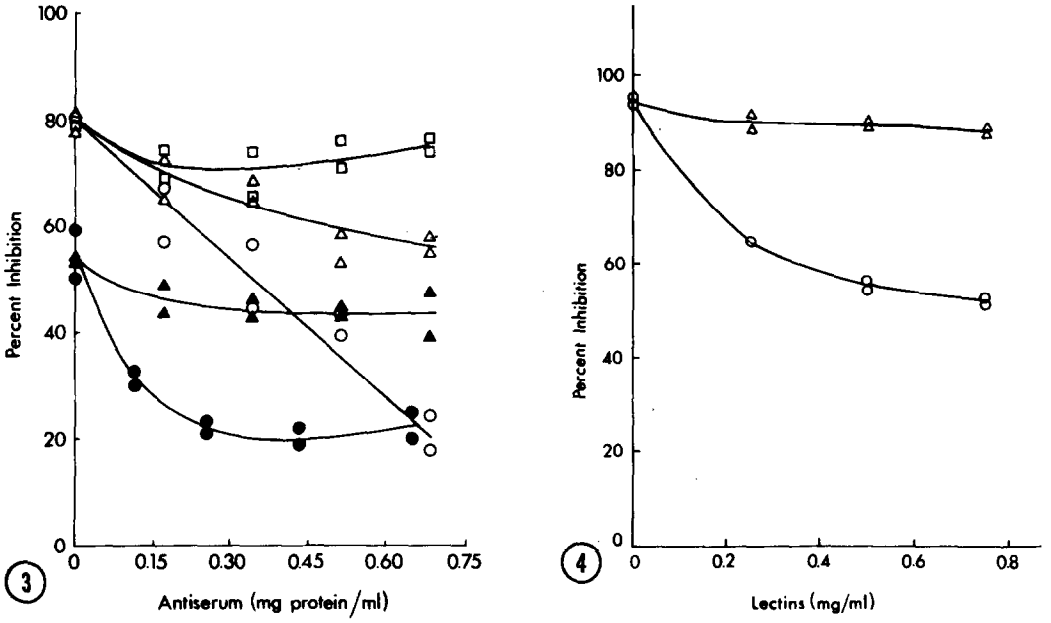

Fig. 3 Antisheep hemolysin neutralization of the inhibitory effect of glycophorins and tryptic fragments. Varied concentrations of hemolysin were mixed to a final volume of 0.1 ml with 10 μg of sheep rbc glycophorin (o), 265 μg of sheep tryptic fragments (▲), 8 μg of rat rbc glycophorin (△), or 15 μg of human rbc glycophorin (●), and allowed to incubate at 30° for 20 min. In addition, pre-immune rabbit serum was used to replace the hemolysin in one incubation with sheep glycophorin (□). Following incubation the mixtures were transferred to 0° and each was mixed with 10 μl (0.03 μg) of PMOW venom. Five min later washed rat rbc's were added to each tube to a final volume of 4 ml (final rbc concentration of 0.625%, v/v) and the % hemolysis ascertained.

Fig. 4 The action of phytohemagglutinin and concanavalin A on the inhibitory effect of glycophorin. Varied concentrations of phytohemagglutinin (o) or concanavalin A (△) were pre-mixed with 6 μg of rat glycophorin to a final volume of 0.1 ml. Following incubation at 30° for 20 min, the mixtures were transferred to 0°, and 0.03 μg (10 μl) of PMOW venom was added to each mixture. Five min later washed rat rbc's were added to each tube to a final volume of 4 ml (0.625%, v/v) to assay the extent of hemolysis.

Glycophorin and band-3 protein are the two major integral proteins of human rbc membranes (10, 11). Glycophorin specifically binds PHA while band-3 binds concanavalin A (Con A). To test the effects of these lectins on the rat glycophorin and band-3 preparations, we added increasing amounts of either lectin to rat glycophorin or band-3 protein before adding PMOW venom. As shown in Fig. 4, the neutralizing effect of glycophorin on venom-induced hemolysis was reversed by PHA whereas Con A had very little

effect. Either PHA or Con A did not reverse the neutralizing effect of rat band-3 protein on the venom-induced hemolysis (data not shown).

Hemolytic action of PMOW venom on DIDS-treated rbc's

DIDS at 2 μM has been reported to completely saturate the band-3 protein and to inhibit anion transports across the membrane of human rbc's (6). In this study, we treated rat rbc's under previously described conditions (6) with DIDS, in concentrations up to 80 μM, and it could not alter the hemolytic sensitivity of the blood cells to PMOW venom.

DISCUSSION

Several lines of evidence obtained in this study indicate that glycophorin, one of the integral membrane glycoproteins of rbc's, is a possible binding site for the PMOW hemolytic factor: (i) Tryptic fragments, which in the case of human rbc's are solely derived from glycophorin (10, 11), in low concentrations can neutralize the hemolytic action of PMOW venom. (ii) The neutralizing effect of glycophorin can be blocked by treating glycophorin with antisheep hemolysin or with PHA, the lectin which specifically binds to glycophorin (3). On the other hand, Con A, which binds to band-3 protein (8) has no significant effect on the inhibitory action of glycophorin. (iii) The hemolytic susceptibility of rbc's derived from various mammals are inversely and linearly related to the I_{50} values for their respective glycophorins. In other words, the most inhibitory glycophorins are those derived from the most sensitive cells.

Glycophorins are typically composed of a central transmembrane hydrophobic domain, an N-terminal carbohydrate bearing hydrophilic domain which is exposed on the external cell surface, and a C-terminal hydrophillic domain which is in contact with the cytoplasm (10, 11). Trypsinization of intact rbc's removed the external, carbohydrate bearing domain from glycophorin (10, 11). Since isolated human tryptic fragments are not inhibitory to the hemolytic action of PMOW venom (Fig. 1), they may function on the in-

tact human rbc to sterically block the binding of the hemolytic factor to the membrane. Trypsinization removes this hindrance and exposes the binding site, thereby increasing the sensitivity of human rbc's to venom-induced hemolysis (Table 1). With rat rbc's the toxin binding site seems to be located on the tryptic fragments of glycophorin, since by themselves they strongly inhibit venom-induced hemolysis (Fig. 1), and their removal from the rat rbc by tryptic cleavage leaves the cells much less sensitive to the hemolytic action of PMOW venom (Table 1).

The nature of the interaction between the hemolytic factor of PMOW venom and glycophorins seems to be hydrophobic. The more hydrophobic human and sheep glycophorin fragments extracted from previously trypsinized rbc's are more potent neutralizers of PMOW venom lytic activity than the intact glycophorins isolated from non-trypsinized rbc's (Fig. 2).

Glycophorins extracted by LIS are usually associated with contaminating acidic phosphatides (12). The neutralizing action of glycophorin on the hemolytic activity of PMOW venom is not likely due to such contaminating lipids. Glycophorin preparations extracted with ethanol and a mixture of chloroform/methanol/acetic acid contained no detectable phosphorus, and were as active in neutralizing the venom's hemolytic activity as non-extracted glycophorins (data not shown).

The carbohydrate residues of glycophorins are probably not involved in the binding of the hemolytic factor of PMOW venom to glycophorin. Tryptic fragments isolated from human rbc's contain all the carbohydrate residues (3, 10, 11), yet they cannot inhibit the venom-induced hemolysis. The inhibitory effect of human glycophorin on the hemolytic activity of PMOW venom was not abolished by treatment with neuraminidase, but was destroyed by pronase (unpublished observations).

Band-3 protein, like glycophorin, is a transmembrane protein (10). Pretreatment of rat rbc's with high concentrations of DIDS or Con A, both of which specifically bind to band-3 protein (6, 8), do not alter the rbc

sensitivity to PMOW venom. Neither is there any apparent relationship between the I_{50} values for band-3 proteins derived from rat, dog and human rbc's and the venom sensitivities of these rbc's (Fig. 2, inset). These negative observations do not support the view that band-3 is involved in the hemolytic action of PMOW venom. Rather, it appears that the nature of the neutralizing effect of isolated band-3 proteins on the hemolytic activity of PMOW venom may be due to nonspecific hydrophobic interactions. Indeed, the hemolytic component of PMOW venom is capable of interacting with hydrophobic detergents (1).

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