Effect of *Bacteroides fragilis* on the human erythrocyte membrane: pathogenesis of Tk polyagglutination

G. INGLIS, G. W. G. BIRD, A. A. B. MITCHELL, G. R. MILNE, AND JUNE WINGHAM

From the Glasgow & West of Scotland Blood Transfusion Service, Law Hospital, Carlisle; The Regional Blood Transfusion Service, Birmingham; and the Department of Bacteriology, Law Hospital, Carlisle

SYNOPSIS Incubation of normal human erythrocytes with the supernatant of centrifuged culture of some strains of *Bacteroides fragilis* results in the exposure of Tk polyagglutination determinants on the erythrocyte membrane. These determinants are present on non papain-labile structures and are probably exposed by an enzyme mechanism. Other strains of *B. fragilis* were found to produce neuraminidase, haemolysins, and protease. It is suggested that early recognition of Tk determinants may be an aid to the diagnosis of *B. fragilis* infection.

Polyagglutination is the agglutination by a variable number of normal sera of altered, defective or otherwise abnormal erythrocytes. This abnormality may be due either to adsorption of extraneous antigen by the erythrocyte or to structural peculiarities of the erythrocyte membrane itself (Bird, 1971).

In vivo polyagglutinability was first described by Levine and Katzin (1938). The membrane determinant responsible was named 'T' by Friedenreich (1930) who showed that it was present in a latent form on all normal human erythrocytes and could be exposed in the presence of 'enzyme-producing bacteria'. It was subsequently shown that the enzyme responsible for T exposure was neuraminidase (Gottschalk, 1960), which specifically cleaves N-acetylneuraminic acid (NANA) from the cell membrane and exposes β -galactosyl determinants (Uhlenbruck *et al*, 1969). Erythrocytes which carry these determinants are polyagglutinable since anti-T is present in most human adult sera. A number of secondary serological characteristics are associated with T antigen exposure. Loss of NANA results in a reduction in the net electronegative charge carried by the erythrocytes (Cook *et al*, 1961) and as a result they may not be aggregated by polycations such as protamine sulphate, Polybrene or polylysine which aggregate erythrocytes carrying a normal electronegative charge (Greenwalt and Steane, 1973). Also erythrocytes carrying exposed T antigens are agglutinated by a lectin extracted from peanuts (Bird, 1964).

In 1972 Bird and Wingham described a new form of polyagglutination, Tk. As shown in table I, the serological properties associated with T and Tk polyagglutination are similar but notable differences are observed in the effect of papain and of protamine sulphate. Earlier this year a second example of Tk polyagglutination was reported (Inglis *et al*, 1975). Preliminary laboratory investigations indicated that the agent responsible for exposure of the Tk determinant was probably the organism *Bacteroides fragilis*. A third example of Tk polyagglutination has since been reported (Bird *et al*, 1975) which demonstrated a definite involvement of this species.

	T Acquired	Tk Acquired
Agglutination by <i>Arachis hypogea</i> lectin	+	+
Agglutination by <i>Dolichos biflorus</i> lectin	-	-
Aggregation by protamine sulphate	-	+
Effect of papain	Some destruction	Enhanced
Chemical basis of specificity	β -Galactose	β -Galactose

Table I Differentiation of T and Tk polyagglutination

In this report we present the results of *in vitro* experiments involving incubation of supernatants of cultures of different strains of *B. fragilis* with normal human erythrocytes and give a possible explanation of the mechanism involved in Tk exposure.

Material

ORGANISMS

Pure cultures of *B. fragilis* from a variety of human sources were incubated at 37°C for three days in cooked meat broth. The supernatant from centrifuged cultures was retained and stored at -20°C until tested. Supernatants from pure cultures of other genera were prepared in an identical manner. These included *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, non-haemolytic streptococci, *Pseudomonas aeruginosa*, *Proteus* species, and *Klebsiella aerogenes*.

ERYTHROCYTES

Normal erythrocytes from blood donors were collected into acid-citrate-dextrose (ACD) anticoagulant. The erythrocytes were washed three times in 0.15 M sodium chloride and examined before use for the absence of polyagglutinability.

PEANUT LECTIN

80 g of peanuts were ground in a coffee grinder and suspended overnight at 4°C in 200 ml of 0.15 M sodium chloride. After centrifugation the aqueous layer was retained as stock reagent. Ten dilutions of stock (1/1 to 1/512) were prepared for use in 0.15 M sodium chloride.

PROTAMINE SULPHATE

Salmine sulphate (BDH Ltd) was prepared as a stock solution (100 mg/dl) in 0.15 M sodium chloride. Ten dilutions, 100 mg/dl-10 mg/dl, were prepared for use in 0.15 M sodium chloride.

NEURAMINIDASE

Neuraminidase, prepared commercially from *Vibrio cholerae* and assayed at 500 units/ml, was purchased from BDH Ltd.

PAPAIN

Papain solution was prepared by the method of Löw (1955) and stored at -20°C before use.

All serological experiments were carried out in 2 × 1/4 inch (50 × 6 mm) polystyrene tubes (Sterilin Ltd).

Methods and Results

TREATMENT OF ERYTHROCYTES WITH NEURAMINIDASE

Neuraminidase was diluted in 0.15 M sodium chloride to give a range of enzyme activity of 40 units/ml to 0.16 units/ml. One volume of washed, concentrated erythrocytes was added to one volume of each neuraminidase dilution and incubated at 37°C for 15 minutes. After centrifugation, the supernatants were recovered and NANA was assayed by the thiobarbituric acid method of Warren (1959). The erythrocytes were washed three times in 0.15 M sodium chloride and resuspended to a final concentration of 3-5% (v/v). Each erythrocyte suspension was tested with dilutions of peanut lectin both in saline and in papain at 4°C for 1 hour (1 vol lectin + 1 vol papain (or saline) + 1 vol erythrocytes); 1 vol of erythrocyte suspension was also tested with 1 vol of protamine sulphate dilutions at 37°C for 30 minutes.

Concentration of Neuraminidase used to treat Erythrocytes (units/ml)	Medium	Dilution of Peanut										NANA (μ moles per ml, RBCs)
		1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	
40	Saline	+++	+++	+++	+++	+++	+++	+++	+++	+	(+)	0.41
	Papain	+	+++	+++	+++	+++	+++	+++	+	-	-	
20	Saline	+++	+++	+++	+++	+++	+++	+++	++	+	(+)	0.36
	Papain	+	+++	+++	+++	+++	+++	+++	++	+	-	
10	Saline	+++	+++	+++	+++	+++	+++	+++	+	(+)	-	0.26
	Papain	+	+++	+++	+++	+++	+++	(+)	-	-	-	
5	Saline	+++	+++	+++	+++	+++	+++	+++	(+)	-	-	0.14
	Papain	(+)	(+)	+++	(+)	+	+	+	-	-	-	
2.5	Saline	+++	+++	+++	+++	+++	+++	(+)	-	-	-	0.07
	Papain	(+)	(+)	(+)	-	-	-	-	-	-	-	
1.25	Saline	+++	+++	+++	+++	(+)	-	-	-	-	-	0.06
	Papain	-	-	-	-	-	-	-	-	-	-	
0.63	Saline	(+)	+	+	(+)	-	-	-	-	-	-	0.02
	Papain	-	-	-	-	-	-	-	-	-	-	
0.32	Saline	-	-	-	-	-	-	-	-	-	-	0.01
	Papain	-	-	-	-	-	-	-	-	-	-	

Table II Erythrocytes treated with neuraminidase—pattern of agglutination with peanut lectin

Results

The results are given in table II. The concentration of NANA released increased with increasing neuraminidase activity, as did the agglutinating activity of the erythrocytes with the peanut lectin. The erythrocytes treated with 0.63 and 1.25 units/ml neuraminidase were agglutinated by lectin in saline but not by lectin in papain. Erythrocytes treated with 2.5 units/ml neuraminidase or greater were agglutinated by lectin both in saline and in papain. As the concentration of neuraminidase increased, the strength of agglutination of the treated erythrocytes in lectin increased in both media but was always greater in saline than in papain for any given erythrocyte suspension. The capacity of the erythrocytes to aggregate in protamine sulphate decreased as increasing concentrations of NANA were cleaved from the cells.

TREATMENT OF HUMAN ERYTHROCYTES WITH SUPERNATANT OF *B. fragilis* CULTURE

The previous experiment was repeated substituting dilutions of *B. fragilis* culture supernatant in 0.15 M sodium chloride for neuraminidase.

Results

The results (table III) demonstrated that a different pattern of agglutination in lectin is obtained with culture-treated cells. Erythrocytes treated with high dilutions of culture supernatant were agglutinated by lectin, but only in the presence of papain. The agglutinating activity of the erythrocytes increased

as the dilution of culture supernatant used to treat the cells decreased. By increasing the incubation time of erythrocytes in neat culture supernatant, cells were obtained which were agglutinated by lectin in papain and in saline. For any given erythrocyte suspension, agglutination by lectin was always greater in papain than in saline. NANA was not detected, either in the free state or as sialopeptide, in the supernatant after culture treatment of the erythrocytes. Aggregation of all the treated erythrocytes was normal in protamine sulphate.

INHIBITION OF PEANUT LECTIN BY *B. fragilis* CULTURE SUPERNATANT

Equal volumes of culture supernatant and dilutions of peanut lectin were incubated for three hours at 4°C. Each dilution of lectin was then tested for inhibition by testing with Tk erythrocytes both in saline and in papain for one hour at 4°C. Inhibition of lectin activity was not observed.

TESTS FOR AGGLUTINATION OF *B. fragilis* ORGANISMS WITH PEANUT LECTIN

A saline suspension of *B. fragilis* organisms was tested at 4°C with peanut lectin both in saline and in papain. The organisms were not agglutinated.

TESTS FOR OTHER STRAINS OF *B. fragilis*

Over 50 different strains of *B. fragilis* were cultured and the centrifuged supernatants examined using the techniques described earlier.

Culture Dilution used to Treat Erythrocyte	Medium	Dilution of Peanut									
		1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$
Neat (60 min)	Saline	+++	+++	+++	+++	++	+	(+)	-	-	-
	Papain	+++	+++	+++	+++	+++	+++	+++	+	(+)	-
Neat (45 min)	Saline	++	+++	+++	+++	+	(+)	-	-	-	-
	Papain	+++	+++	+++	+++	+++	+++	+++	+	(+)	-
Neat (30 min)	Saline	(+)	(+)	(+)	-	-	-	-	-	-	-
	Papain	++	+++	+++	+++	+++	+++	+++	+	(+)	-
Neat (15 min)	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	++	+++	+++	+++	+++	+++	+++	+	(+)	-
1/2	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	++	+++	+++	+++	+++	+++	+++	(+)	-	-
1/4	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	+	+++	+++	+++	+++	+++	++	(+)	-	-
1/8	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	+	+++	+++	+++	+++	++	+	-	-	-
1/16	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	(+)	+	+++	+++	+++	+	(+)	-	-	-
1/32	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	-	(+)	+	+	(+)	-	-	-	-	-
1/64	Saline	-	-	-	-	-	-	-	-	-	-
	Papain	-	-	-	-	-	-	-	-	-	-

Table III Erythrocytes treated with *Bacteroides fragilis* culture—pattern of agglutination with peanut lectin

Results

Five different reaction patterns were observed:

- (1) Haemolysis of the erythrocytes
- (2) Exposure of T determinants
- (3) Exposure of Tk determinants
- (4) Exposure of (Tk + T) determinants
- (5) Reduction of the electronegative charge without exposure of polyagglutination determinants, ie, similar to protease treatment of erythrocytes.

Discussion

A concept of layers of antigen activity within erythrocyte membranes has been postulated by Uhlenbruck and Wintzer (1970), who found that N-acetylneuraminic acid can be detected both on glycoprotein and on glycolipid structures and that these structures are very differently distributed in the membrane. The data presented in table II support a layer hypothesis for T determinants and suggest that there are at least two areas of T activity, one on the outer surface of the membrane, carried on papain-labile structures, and the other on a lower layer of non papain-labile structures. The concentration of neuraminidase available governs which determinants are exposed, papain-labile structures appearing to carry the dominant substrate since only these are exposed by low concentrations of neuraminidase. The conflicting reports in the literature of papain lability of T determinants are also explained by this model.

The results in table III indicate that Tk determinants are found only on non papain-labile structures. When only small numbers of determinants are exposed, the use of papain is necessary to detect them. By sufficiently increasing the site density of exposed Tk determinants, the erythrocytes may be agglutinated by lectin without the aid of papain. The addition of papain enhances agglutination, suggesting that removal of surface peptide facilitates the reaction with lectin.

Tk determinants are similar to but not identical with T determinants. Both have similar β -galactosyl determinants which are quite different from other β -galactosyl determinants on the red cell membrane surface (Pardoe *et al*, 1971) so that erythrocytes carrying exposed T or Tk determinants are agglutinated by the peanut lectin. However, Bird and Wingham (1972) have shown that normal human serum absorbed with neuraminidase-treated erythrocytes can agglutinate Tk erythrocytes. Non identity is further demonstrated by the fact that exposure of Tk determinants is not accompanied by the release of NANA from the erythrocyte membrane.

It is probable that the mechanism responsible for

Tk exposure is enzymatic since an attempt to inhibit the serological activity of peanut lectin by incubation with culture supernatant was unsuccessful, as was the attempt to identify Tk determinants on *B. fragilis* organisms themselves.

Not all strains of *B. fragilis* induce Tk exposure on normal erythrocytes. Some produce a neuraminidase and expose T determinants on normal erythrocytes. Others expose both T and Tk determinants. Haemolysin and protease-like activity were also observed in a few strains.

Although only a relatively small number of genera were tested, *B. fragilis* was the only species capable of inducing Tk exposure on erythrocytes. On both occasions in which Tk polyagglutination was associated *in vivo* with *B. fragilis* infection, death resulted. It may therefore be that Tk polyagglutination indicates a poor prognosis. On the other hand, since only a minority of patients with infection require transfusion therapy, the Tk phenomenon may not be as rare as statistics indicate, in which case early identification of Tk exposure may be useful as an aid to the diagnosis of *B. fragilis* infection.

References

- Bird, G. W. G. (1964). Anti-T in peanuts. *Vox Sang. (Basel)*, **9**, 748-749.
- Bird, G. W. G. (1971). Erythrocyte polyagglutination. *Nouv. Rev. franc. Hémat.*, **11**, 885-896.
- Bird, G. W. G. and Wingham, J. (1972). Tk: a new form of red cell polyagglutination. *Brit. J. Haemat.*, **23**, 759-763.
- Bird, G. W. G., Wingham, J., Inglis, G., and Mitchell, A. A. B. (1975). Tk polyagglutination in *Bacteroides fragilis* septicaemia. (Letter). *Lancet*, **1**, 286.
- Cook, G. M. W., Heard, D. H., and Seaman, G. V. F. (1961). Sialic acids and the electrokinetic charge of the human erythrocyte. *Nature (Lond.)*, **191**, 44-47.
- Friedenreich, V. (1930). *The Thomsen Hemagglutination Phenomenon*. Levin and Munksgaard, Copenhagen.
- Gottschalk, A. (1960). *The Chemistry and Biology of Sialic Acids and Related Substances*. Cambridge University Press, Cambridge.
- Greenwalt, T. J. and Steane, E. A. (1973). Quantitative haemagglutination. VI. Relationship of sialic acid content of red cells and aggregation by Polybrene, protamine and poly-l-lysine. *Brit. J. Haemat.*, **25**, 227-237.
- Inglis, G., Bird, G. W. G., Mitchell, A. A. B., Milne, G. R., and Wingham, J. (1975). Erythrocyte polyagglutination showing properties of both T and Tk, probably induced by *Bacteroides fragilis* infection. *Vox Sang. (Basel)*, **28**, 314-317.
- Levine, P. and Katzin, E. M. (1938). Temporary agglutination of red blood cells. *Proc. Soc. exp. Biol. (N.Y.)*, **39**, 167-169.
- Löw, B. (1955). A practical method using papain and incomplete Rh-antibodies in routine Rh blood-grouping. *Vox Sang. (Basel)*, (O.S.), **5**, 94-98.
- Pardoe, G. I., Bird, G. W. G., Uhlenbruck, G., and Anstee, D. J. (1971). Immunochemistry of galactosyl groups of cell membranes. In *The Role of Lymphocytes and Macrophages in the Immunological Response*, edited by D. C. Dumonde, pp. 91-98. Springer, Berlin and New York.

- Uhlenbruck, G., Pardoe, G. I., and Bird, G. W. G. (1969). On the specificity of lectins with a broad agglutination spectrum. II. Studies on the nature of the T-antigen and the specific receptors for the lectin *Arachis hypogaea* (ground nut). *Z. Immun.-Forsch.*, **138**, 423-433.
- Uhlenbruck, G. and Wintzer, G. (1970). Topochemical

arrangement of neuraminic acid containing receptors within the cell membrane. In *Blood and Tissue Antigens*, edited by D. Aminoff, pp. 289-305. Academic Press, New York and London.

- Warren, L. (1959). The thiobarbituric acid assay of sialic acids. *J. biol. Chem.*, **234**, 1971-1975.

The November 1975 Issue

THE NOVEMBER 1975 ISSUE CONTAINS THE FOLLOWING PAPERS

- Smallpox eradication—the final battle** D. A. HENDERSON
- A serodiagnostic test for tuberculosis** A. C. NICHOLLS
- The VDRL test in a blood transfusion service** R. W. BEAL AND D. J. MERRY
- Mechanized blood grouping: a hospital trial using an 8-channel grouping machine** G. C. JENKINS, R. G. FEWELL, M. J. LLOYD, JEAN BROWN, J. JUDD, R. S. LANE, AND W. J. JENKINS
- α d and α y subtypes of hepatitis B antigen in a case of hypogammaglobulinaemia** J. W. M. LAWTON, R. HOPKINS, I. C. PATERSON, AND P. C. DAS
- Variations in the level of a pregnancy-associated α -macroglobulin in patients with cancer** W. H. STIMSON
- Fibrinolytic activity in malignant disease** J. A. N. RENNIE AND D. OGSTON
- Plasma erythropoietin assay in patients with chronic renal failure** SYLVIA DAVIES, EVELINE GLYNNE-JONES, MARGARET BISSON, AND P. BISSON
- Renal dysplasia in nephrectomy specimens from adolescents and adults** C. FISHER AND J. F. SMITH
- Necropsy study on glomerulonephritis in the elderly** P. R. POTVLIEGE, G. DE ROY, AND F. DUPUIS
- Interpretation of respiratory tract histology in deaths** E. TAPP, D. M. JONES, AND J. O'H. TOBIN
- Quantitative study of uterine curettage in the menstrual cycle** B. BISWAS AND J. A. H. FINBOW
- Laboratory procedures for the isolation of *Chlamydia trachomatis* from the human genital tract** P. REEVE, J. OWEN, AND J. D. ORIEL
- Stability of thyroxine and triiodothyronine in biological fluids** LYNN NYE, T. H. YEO, VIVIAN CHAN, D. GOLDIE, AND J. LANDON
- Technical methods**
A standardized Romanowsky stain prepared from purified dyes P. N. MARSHALL, S. A. BENTLEY, AND S. M. LEWIS
- Modifications to improve the reliability of the Coulter 'S'** P. F. J. NEWMAN, E. ROBERTSON, AND M. J. MUIR
- Letter to the Editor**
- Book reviews**

Copies are still available and may be obtained from the PUBLISHING MANAGER, BRITISH MEDICAL ASSOCIATION, TAVISTOCK SQUARE, LONDON, WC1H 9JR, price £2.00