33 Polyagglutination and Cryptantigens

33.1 Introduction

Polyagglutination was defined by Bird [1] as, 'The agglutination of red cells, irrespective of blood group, by many sera, the abnormality being a property of the red cells and not the sera'. Microbial polyagglutination generally results from the effect of bacterial or viral glycosidases revealing concealed antigenic determinants, called cryptantigens. These enzymes catalyse the cleavage of terminal monosaccharides or acetyl groups from the oligosaccharide chains of membrane glycoproteins or glycolipids. Polyagglutination occurs because of IgM antibodies against these cryptantigens present in the serum of most people; sera from those individuals with polyagglutinable red cells usually lack the corresponding antibody. Somatic mutation resulting in incomplete biosynthesis of oligosaccharides may lead to a more persistent expression of cryptantigen. Another type of polyagglutination arises from the inheritance of very rare antigens, which are detected by antibodies in most adult human sera. A classification of polyagglutination is provided in Table 33.1. For reviews on polyagglutination see [1–7].

Lectins are non-immunoglobulin extracts from plants and occasionally animals. They have proved invaluable in the identification and classification of cryptantigens and polyagglutination. Some of the most useful lectins are listed in Table 33.2.

33.2 Acquired polyagglutination and the cryptantigens involved

33.2.1 Microbial polyagglutination

Microbial polyagglutination is associated with septicaemia, bowel or respiratory tract infections, wound infections, and conditions, such as tumours or obstructions, that compromise the integrity of the bowel wall and permit entry of microbial enzymes into the blood [1,2,5–7,17]. Occasionally microbial polyagglutination is detected in apparently healthy individuals, presumably because of subclinical infection or an undetected intestinal lesion. Before microbial enzymes can modify red cell membrane structures they must be present in sufficient quantity to neutralise plasma enzyme inhibitors. Cryptantigens are quite commonly detectable with lectins, but not with human sera, on the red cells of patients with various infections, sometimes before the infection has been diagnosed. Microbial polyagglutination is usually transient, the polyagglutinability of the cells diminishing as infection abates.

Red cells may also become polyagglutinable *in vitro*, either as a result of bacterial contamination of blood samples or by the use of enzymes isolated from micro-organisms.

33.2.1.1 T polyagglutination

The Hübener–Thomsen–Friedenreich phenomenon [18] or T activation of red cells results from microbial

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sialidases catalysing the cleavage of sialic acid residues from the *O*-linked disialotetrasaccharides of the major red cell sialoglycoproteins, glycophorins A and B, and from other glycoproteins and glycolipids. The T determinant (CD176) is basically terminal D-galactose in β1,3 linkage with GalNAc, the core 1 *O*-glycan (Table 33.3 and see Chapter 3). T exposure causes depression of M and N antigens [5] and the reduction in cell surface charge makes T-active cells agglutinable by *Glycine soja* lectin [14]. Sialidases from bacteria such as *Clostridium perfringens*, *Vibrio cholerae*, and pneumococci, and from the influenza virus, have all been responsible for *in vivo* T cryptantigen exposure. T activation is more common in infants than in adults and is particularly associated with paediatric intestinal disorders [17,19,20].

In 1964, Bird [8] found that extracts of peanuts, *Arachis hypogaea*, made a powerful anti-T lectin. The term 'peanut-positive polyagglutination' now refers to the exposure of any of five cryptantigens, T, Tk, Th, Tx, or Tr, that bind *Arachis hypogaea* lectin (Table 33.2). Red cells of 52 (0.5%) of 9672 hospital patients were agglutinated by *Arachis hypogaea* lectin, but in only one patient were the cells polyagglutinable [21].

Almost all normal human sera agglutinate T-activated red cells, but only a very small proportion (2% [22]) are haemolytic *in vitro*. This haemolytic activity is usually weak and dependent on the degree of desialylation [22]. Monoclonal antibodies with T specificity have been produced following the immunisation of rodents with desialylated red cells [23–25]. Some monoclonal anti-Le^a cross-react with T-activated Le(a–) red cells [26].

T polyagglutination is occasionally accompanied by haemolysis [27]. Whether this is due to complement fixing anti-T in the patient's serum or to an effect of the associated infection is unclear. Haemolytic-uraemic syndrome in children following pneumococcal infection may be caused by the effect of bacterial sialidase; T activation of red cells, platelets, and renal glomeruli could be responsible for the characteristic symptoms of haemolytic anaemia, thrombocytopenia, and renal failure [28–30]. Transfusion of plasma or whole blood containing anti-T has been implicated as a cause of severe haemolytic reactions in infants with necrotising enterocolitis

Table 33.1 Classification of polyagglutination.

Acquired
Microbial
- Cryptantigens uncovered by microbial enzymes
  - T, Tk, Th, Tx, Acquired B
- Adsorption of bacteria or bacterial products onto cell surface

Non-microbial (persistent)
- Biosynthetic blockage resulting from somatic mutation
  - Tn, ?Th

Inherited
- Sd(a++) (Cad), CDA II, NOR, Hyde Park

Undetermined
- VA, Tr

Table 33.2 Reactions of lectins with cryptantigens and with Sd(a++) and Hyde Park polyagglutinable cells.

Lectins	T	Th	Tk	Tx	Tn	Sd(a++)	Hyde Park	Tr	References
Arachis hypogaea	+	+	+	+	–	–	w	+	[8]
Vicia hyrcanica	+	+	+		–	–	+		[9]
Vicia cretica	+	+	–	–	–	–	w		[10]
Vicia villosa	+	–	–		+	+	+	+	[11]
Griffonia simplicifolia (GSII)	–	–	+	–	–	–	+	+	[12]
Medicago disciformis	+	+	–	–	–		+		[13]
Dolichos biflorus (B and O only)	–	–	–	–	+	+	–	–	[14]
Salvia sclarea	–	–	–	–	+	–	–	+	[15]
Salvia horminum	–	–	–	–	+	+	w	+	[15]
Leonurus cardiaca	w	–	–	–	–	+	–		[16]
Glycine soja	+	–	–	–	+	+	+/–		[14]

w, weak agglutination; +/–, reactive with strong examples only.

Table 33.3 *O*-linked oligosaccharides of sialoglycoproteins from normal red cells and from T- and Tn-activated cells. See Table 2.4 for abbreviations.

Normal	NeuAcα2→3Galβ1→3GalNAc—Ser/Thr 6 ↑ NeuAcα2
T	Galβ1→3GalNAc—Ser/Thr
(core 1)	
Tn	GalNAc—Ser/Thr
Sialyl-Tn	GalNAc—Ser/Thr 6 ↑ NeuAcα2

(NEC) and T-activated red cells, though this complication is rare [17,31].Whether patients likely to have T-activated cells, such as children with NEC, should be transfused with plasma, is controversial [17,22], and plasma exchange may be considered beneficial in extreme cases [32].

Leucocytes and platelets of individuals with T-polyagglutinable red cells also express T antigen [33].

33.2.1.2 Th polyagglutination

Th polyagglutination is distinguished from T polyagglutination by the use of lectins (Table 33.2) and probably represents a weak expression or early stage of T activation caused by sialidase from *Corynebacterium aquaticum* [34,35]. Sialidases from *Vibrio cholerae* used under conditions of particularly mild hydrolysis will also produce Th activity, *in vitro*. Release of sialic acid below a critical quantity (20 μg per 10^{10} red cells) leads to Th activity, whereas liberation of greater quantities of sialic acid results in T polyagglutinability [35]. It is possible that Th activity represents the removal of only one sialic acid residue from the tetrasaccharide shown in Table 32.3, exposing terminal Gal, but not the T disaccharide (Galβ1→3GalNAc).

Red cells from 22 of 200 cord samples were Th-activated (but not polyagglutinable); in six of these the mother's red cells were also Th-active [36]. A patient with Th polyagglutination and peritonitis arising from a perforated colonic tumour died as a result of severe intravascular haemolysis [37].

It is possible that a persistent form of Th polyagglutination resulting from incomplete biosynthesis also occurs. Persistent Th activation in the absence of infection was identified in five of seven children with congenital hypoplastic anaemia [38] and in a patient with myelodysplasia whose red cells were also Tn-active [39].

33.2.1.3 Tk polyagglutination

Tk polyagglutination [40] is most readily distinguished from T polyagglutination by the use of GSII, a Tk-specific lectin isolated from the seeds of *Griffonia simplicifolia* [12]. Expression of Tk is enhanced by papain treatment and Tk-activated cells are not sialic acid deficient [40]. *Vicia hyrcanica* seeds contain separable T- and Tk-specific lectins [9]. Monoclonal anti-Tk has been produced by immunising mice with endo-β-galactosidase-modified red cells [41].

Tk polyagglutination is usually associated with *Bacteroides fragilis* infection [42], but *Serratia marcescens*, *Candida albicans*, *Aspergillus niger* and, in children, invasive pneumococcal infection, have also been responsible [3,4,43]. Endo- and exo-β-galactosidases secreted by these pathogens cleave oligosaccharides at Galβ1→4GlcNAc linkages on ABH and Ii active blood group substances (Tables 2.3, 2.5, and 25.1) exposing terminal GlcNAc residues, the Tk receptor [44,45]. Tk-transformed red cells may have reduced expression of ABH and Ii antigens [46,47]. GSII lectin is inhibited by GlcNAc [12,48] and monoclonal anti-Tk was adsorbed by GlcNAcβ1→3Galβ1→4Glcβ–R coupled to silica beads [41]. Endo-β-galactosidases from *B. fragilis, Escherichia freundii*, and *Flavobacterium keratolycus* have been used to produce Tk-activated red cells *in vitro* [41,45,49].

Tk polyagglutination is often associated with acquired B polyagglutination (Section 33.2.1.5) and with T activation in pneumococcal infections in infants [43].

33.2.1.4 Tx cryptantigen

Tx was identified on the red cells of children with pneumococcal infections and could be produced, *in vitro*, by the action on red cells of culture supernatant from pneumococci derived from these patients [50]. Tx is distinguished from T by *Vicia cretica* lectin (Table 33.2). Tx is only slightly weakened by papain treatment [50]. A persistent form of Tx polyagglutination was detected over a period of 5 years in a woman with myelodysplastic syndrome and no symptoms of infection [51].

33.2.1.5 Acquired B

Acquired B phenomenon is described in detail in Section 2.13.1. It is caused by microbial deacetylases converting GalNAc of an A determinant to galactosamine, which

resembles Gal closely enough for the cells to be agglutinated by some anti-B reagents. Acquired B is usually associated with a unique polyagglutination, but is often also associated with Tk and occasionally T or Th polyagglutination.

33.2.2 Non-microbial polyagglutination

33.2.2.1 Tn polyagglutination

In Tn polyagglutination [52] only a proportion of the red cells are polyagglutinable: between 30 and 90% are agglutinated by anti-Tn, the remainder are Tn-negative. Like T, Tn is located on *O*-linked oligosaccharides of red cell glycoproteins, chiefly glycophorins A and B [53]. The Tn determinant (CD175) is GalNAc attached to serine or threonine (Table 33.3) [51–55]. The GalNAc is sometimes sialylated, to form sialyl-Tn (CD175s, Table 33.3) [56].

Tn is exposed by defective oligosaccharide biosynthesis [57,58]. Tn is the biosynthetic precursor of T (core 1), which is a precursor of the disialotetrasaccharide typical of red cell sialoglycoproteins (Table 33.3). In Tn-polyagglutinable cells, conversion of the Tn monosaccharide to the T disaccharide does not occur because of deficiency of T synthase, the core 1 β-3-D-galactosyltransferase [59,60]. This enzyme deficiency arises from a somatic mutation within a pluripotent haemopoietic stem cell and subsequent clonal expansion and proliferation of populations of Tn+ red cells, platelets, granulocytes, and lymphocytes [61–63]. Tn-active red cells are not totally deficient in T synthase as some normal tetrasaccharides are present on the glycophorin molecules [64].

The somatic mutations responsible for Tn phenotype do not occur in *C1GALT1*, the gene encoding T synthase on chromosome 7p21.3, but in *C1GALT1C1*, the gene encoding Cosmc, a molecular chaperone required for effective functioning of T synthase [65–67]. Cosmc interacts with denatured, but not native, T synthase to form a non-covalent and reversible complex that results in the acquisition of T-synthase activity [68]. Four Cosmc mutations have been shown to be responsible for the Tn phenotype in different individuals: 3G>C, Met1Ile; 202C>T, Arg68stop; 454G>A, Glu152Lys; and 577T>C, Ser 193Pro. In one other Tn individual no Cosmc cDNA was detected [66,67]. Cells of the Jurkat human lymphoblastoid T-cell line express Tn and have a mutated Cosmc gene with a deletion introducing a premature stop codon [65]. Transfection of Jurkat cells with Cosmc cDNA restores T-synthase activity and ablates Tn expression, but transfection of Cosmc cDNA containing any of the Tn mutations has no affect on Tn expression [66, 67]. The Cosmc gene is X-linked, located at Xq24 [65]. Owing to the presence of a Y-chromosome in males and X-chromosome inactivation in females, somatic cells contain only one active X-chromosome and consequently only one active Cosmc gene. If a similar process occurs in Tn phenotype as that in PNH (see Section 19.5), then cytotoxic T cells targeting the normal *O*-glycans of blood cells would be expected in individuals having large populations of Tn-positive red cells. Experimental ablation of the Cosmc gene in mice results in embryonic death, with Tn expressed on every observable cell of the embryo [69].

Tn-active red cells have reduced sialic acid content [53,70] and depressed M and N antigens and T cryptantigen [54,71,72]. Tn is destroyed by papain treatment of red cells [70,73].

The immunodominant monosaccharide of Tn is GalNAc, so Tn-activated red cells are agglutinated by lectins that also agglutinate group A cells, such as *Dolichos biflorus* and *Helix pomatia* [57,70,73]. *Salvia sclarea* lectin is more specific for Tn (Table 33.2) [15]. Tn cells are more strongly agglutinated by human sera containing anti-A than those lacking it [70]. Many murine monoclonal Tn antibodies have been produced, though they may cross-react with other carbohydrate epitopes [74], including sialyl-Tn [75]. An scFv–Fc fusion protein, produced by employing a simultaneous positive and negative selection strategy with Tn and A antigens, respectively, had strict Tn specificity [76]. All Tn antibodies and lectins react only with the Tn+ population of cells giving the characteristic mixed field pattern of agglutination with red cells of individuals with Tn polyagglutination.

Tn polyagglutination is often associated with haemolytic anaemia, leucopenia, and thrombocytopenia [27] and, on occasion, is detected in healthy blood donors [70,77]. Although usually persistent, Tn polyagglutination has been known to recede [58]. There are three reports of transient Tn polyagglutination in newborns, possibly resulting from late development of full T-synthase activity [78–80]. Flow cytometry with monoclonal anti-Tn revealed less than $1x10^{-6}$ Tn red cells in the peripheral blood of healthy donors [81].

33.2.3 T, Tn, sialyl-Tn, and Tk in malignancy

T and Tn are cryptantigens on epithelial tissues, in the carbohydrate chains of glycoproteins and glycolipids. Springer and others have shown that T, Tn, or sialyl-Tn are exposed on about 90% of primary and metastatic

carcinomas, probably as a result of incomplete biosynthesis [82]. All human tumour cells examined that express Tn and sialyl-Tn harbour mutations in the Cosmc gene, resulting in loss of T-synthase activity [83]. Increased density of Tn over T is an indicator of high metastatic potential of the tumour. Immunotherapy with a vaccine derived from T- and Tn-activated red cells has been successful in preventing recurrence of breast cancer and has a potential for wider application (reviewed in [84,85]). Monoclonal anti-Tk reacted with 48% of human colorectal carcinomas [41].

Reports of patients with Tn polyagglutination and concurrent acute myeloid leukaemia (AML), or who subsequently developed leukaemia, led Ness *et al.* [86] to propose that Tn polyagglutination may represent a preleukaemic state and that careful clinical observation of individuals with Tn polyagglutination would be circumspect. Polyagglutination vanished during chemotherapy [58,86]. Tn polyagglutination has also been associated with myelodysplasia [39,77].

33.3 Inherited polyagglutination

33.3.1 Sd(a++) (Cad)

Sd^a is a red cell antigen with an incidence of about 91% and of variable strength. Red cells of the very rare individuals with an extra strong form of Sd^a antigen (referred to as Sd(a++) or Cad) are polyagglutinable. The immunodominant structure of Sd^a is GalNAc in β-linkage with Gal. Sd(a++) red cells are agglutinated by *Dolichos biflorus* and *Helix pomatia* lectins, but can be distinguished from Tn cells by *Salvia sclarea* and *Leonurus cardiaca* (Table 33.2). Sd^a and Sd(a++) are described in detail in Chapter 31.

33.3.2 Congenital dyserythropoietic anaemia type II (CDA II or HEMPAS)

CDA II (or hereditary erythroblastic multinuclearity with a positive acidified serum lysis test, HEMPAS) is a rare, autosomal-recessive syndrome found predominantly in southern Italy and Central Europe [87,88]. Most patients have a mild anaemia, although some become transfusion-dependent. Red cells of patients with CDA II are agglutinated at 20°C, or lysed at 37°C, by complement fixing IgM antibodies in about one third of normal sera [89,90]. CDA II red cells have elevated i, normal I, and depressed H [89–92]. They have reduced levels of *N*-glycosylation of cell surface glycoproteins, particularly band 3 and band 4.5 (glucose transporter), which contain repeating *N*-acetyllactosamine (Galβ1→4GlcNAc) units [93], revealing a cryptantigen responsible for the polyagglutination and resulting in excess production of i-active linear *N*-acetyllactosaminylceramides. In addition, 10–35% of erythroblasts in CDA II are binucleated and some red cells have a second membrane close to the outer membrane [87].

CDA II is caused by mutations in *SEC23B*, a gene comprising 20 exons and located on chromosome 20p11.23 [94,95]. *SEC23B* encodes a component of the COPII coat protein complex [96], which is involved in protein-trafficking through membrane vesicles. Abnormalities of *SEC23B* may disturb endoplasmic reticulum-to-Golgi trafficking and interfere with sugar transporters and glycosyltransferases, affecting glycosylation pathways [97]. Homozygosity and compound heterozygosity for numerous *SEC23B* missense or inactivating mutations result in CDA II [94,95,98–100]. Homozygosity or compound heterozygosity for two inactivating mutations has been found only once [100], suggesting that complete lack of the protein could be incompatible with life; compound heterozygosity for a missense and an inactivating mutation tends to result in more severe symptoms than homozygosity or compound heterozygosity for missense mutations [98]. The most common mutations encode Glu109Lys and Arg14Trp and represent 32% and 19%, respectively, of mutations identified [100]. CDAII patients exhibit 40–60% decrease in *SEC23B* mRNA levels, and the patient with a nonsense mutation and a splice site mutation had 30% mRNA level [100].

33.3.3 NOR polyagglutination

NOR is a very rare form of polyagglutination found in only two families. It results from the expression of abnormal glycolipids resulting from a mutation in *A4GALT*, the gene encoding the enzyme responsible for biosynthesis of P1 and P antigens. Consequently, NOR antigen is P1PK4 and is described in Section 4.5.

33.3.4 Hyde Park polyagglutination

The association between a unique form of polyagglutination associated with a rare haemoglobin variant in a large South African family of mixed ethnicity remains a puzzle. Thirty-five members of the family were studied: 12 had the variant haemoglobin M-Hyde Park and polyagglutinable red cells; 23 had neither [101]. Polyagglutination had not been observed previously in association with haemoglobin M-Hyde Park.

Group O, N+ Hyde Park red cells show the following serological characteristics [101,102]:
1 agglutination with a minority of normal human sera (7 of 40);
2 enhanced agglutination with *Vicia graminea* and *Ulex europaeus* lectins and with human anti-I and -i, but normal strength agglutination with rabbit and monoclonal anti-N;
3 agglutination by monoclonal anti-Tn, but not by Tn-specific *Salvia sclarea* lectin;
4 agglutination by *Glycine soja*, *Sophora japonica*, and *Arachis hypogaea* (weakly), lectins that detect desialylated *O*-glycans; and
5 agglutination by the GlcNAc-specific lectins *Vicia hyrcanica* and GSII (weakly) (Table 33.2).
These serological characteristics, together with results of biochemical analyses, suggest that the polyagglutination results from two unrelated anomalies, one associated with heterogeneity of sialylation of the *O*-glycans of glycophorin molecules and the other associated with exposed GalNAc residues on the *N*-glycans of band 3 and band 4.5 [103].

33.4 Polyagglutination of undetermined status

33.4.1 VA polyagglutination

VA polyagglutination is very rare. The red cells are not agglutinated by *Arachis hypogaea* or *Dolichos biflorus* lectins, but give a mixed-field agglutination with *Helix pomatia* lectin [103,104]; they also give a characteristic stippled appearance in immunofluorescence with *H. pomatia* lectin [104]. An associated depression of H antigen has led to the suggestion that microbial α-fucosidase could be responsible for VA exposure [103, 105]. In the original case VA polyagglutination was persistent and associated with haemolytic anaemia [103,104]; in the only other reported example, VA-active red cells were also Tk active [105].

33.4.2 Tr polyagglutination

Tr polyagglutination has been found in one individual [106]. Tr red cells gave a unique pattern of reactions with a panel of lectins (Table 33.2) and reacted with monoclonal anti-T + Tn and -Tk. Results of tests with lectins, reduced periodic acid-Schiff staining of glycophorins A, B, and C, and increased electrophoretic mobility of band 3 suggested a reduction in sialylation of both *N*- and *O*-glycans, exposing β1→4Gal and β1→3Gal residues. It is feasible that Tr results from a defective glycosyltransferase, probably an α1,6-sialyltransferase. If so, that may also account for a bleeding defect, probably resulting from reduced platelet glycoprotein Ib levels, and for other defects suggesting a connective tissue disorder.

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