Agglutination of erythrocytes using lectin-labeled spacers

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Summary. The mechanism of agglutination of human erythrocytes by lectin, studied using lectin-labeled gold granules as spacers (5–64 nm in size), was found to depend upon the size of the spacer but not upon the number and the mobility of the receptors.

Recently we have developed a new method for locating cell surface receptors by transmission and scanning electron microscopy using gold markers of various sizes[1,2]. It was observed that marking of cell membrane glycoproteins by certain lectin-labeled gold granules occurred only when the size of the probe was below a certain limit[2-4]. I have now studied the agglutination of human red blood cell, O Rh^+ (RBC) by lectin-labeled gold granules as function of the probe size, the charge of the cell suface and the mobility of the lectin receptors. This new method gives valuable information on the spacing of glycoprotein brushes on the cell surface and on the mechanism of RBC agglutination.

The agglutination of cells is thought to be determined by factors such as the membrane deformability, the number of receptors, their distribution and their mobility in the membrane, the valency of the agglutinating agent and its size, the electric charge of the cell surface, the metabolic state of the cell[5]. Spacing of the ektoprotein and glycoprotein brushes of the cell surface has also been thought to influence cell agglutinability[6]. However, clustering of lectin receptors was recently dismissed as being an important factor in the agglutinability of RBC[7,8].

Gold granules of various sizes were prepared either by reduction of gold chloride with white phosphorus or by reduction of 0.01% $HAuCl_4$ with 1% sodium citrate as described earlier[1,2]. Data on the gold granules are given in table 1. As a model, the number of protein molecules bound by 1 gold granule was determined with beef liver catalase as described earlier[1]. The granules were labeled[1,4] with concanavalin A (Con A, Miles Laboratories) which is specific for α-D-glucose, α-D-mannose and related saccharide groups[9], *Ricinus communis* lectin (RCA_I, Sigma, type II) which is specific for D-galactose residues[10]; wheat germ agglutinin (WGA) which is specific for N-acetyl-D-glucosamine containing residues[11,12] and soya bean agglutinin (SBA, Pharmacia Fine Chemicals) which is specific mainly for N-acetyl-D-galactosamine residues[13]. In RBC membrane, Con A binds exclusively to Band 3[14], WGA to the sialoglycoproteins[15] and RCA_I to all major glycoproteins[10,14].

The data in table 2 on the agglutination of human RBC by lectin-labeled gold granules reflected 2 effects: the influence of the cell surface charge and the size of the probe (spacer) on the binding and the agglutination reactions. These results show that binding of the probe by RBC occurred when its size was below 17 nm for Con A, 50 nm for SBA and 64 nm for RCA_I. However WGA-markers up to 64 nm in size (even 75 nm as reported earlier[2]) were all well bound by human RBC. These results indicate that receptors for Con A, SBA and RCA_I are located between glycoprotein brushes (or clusters of brushes) of increasing spacing, respectively.

Such differences in binding are not caused either by the mass of the granules, since spacing of lectin receptors was different on hepatocyte membrane[4], milk fat globule membrane[3] and fibroblasts (unpublished), or by desorption of the lectins, since they are tightly bound by gold granules[1,2], or by a low association constant of the markers. As indicated earlier, the association constant of such markers with RBC is very high and is the result of multivalent interactions between receptors and the markers[1,2]. Depending upon its size, 1 gold granule may cover many receptors[1]. Although the number of lectin molecules bound per granule was not determined, it must be greater than that of catalase molecules (table 1) whose mol. wt (250,000) is larger than that of either Con A (100,000) or RCA_I and SBA (120,000).

Electron microscopical observations of thin sections indicated that all Au_5 markers were bound by RBC (results not shown). However, agglutination was not observed with WGA- and SBA-Au_5 since binding of these markers is probably faster than agglutination, which is rate-determining in these conditions[7]. In fact WGA and SBA appear to bind considerably faster than Con A to RBC[7,16]. The repulsion of RBC due to the negative charge of the cell surface had little effect on agglutination by WGA when the spacer was 32 ± 10 nm in size (only 70 granules per cell in the assay caused agglutination). With the larger WGA-Au markers, this minimal number increased owing probably to shearing of the granules during agitation. Although the number of granules bound to RBC is not known, it must be below 70 when agglutination is first observed.

It has been shown that neuraminidase removes 95–100% of sialic acid from the surface of RBC[17,18] and has little influence on the number of Con A and WGA binding sites ($1.2–1.7\times10^5$, $7.9–8.2\times10^6$, respectively[7,15]). However, the

Table 1. Data on gold granules

Granules	Citrate added (ml/100 ml 0.01% $HAuCl_4$)	Average size (nm)	Number of granules per ml for an extinction of 1.0 ($\times 10^{10}$)[a]	Number of catalase molecules bound per gold granule
Au_5	-[b]	5.2± 2.5[c]	4110	1
Au_{17}	4[d]	17 ± 3	103	9
Au_{26}	2	26 ± 5	22.6	20
Au_{32}	1.5	32 ±10	11.4	27
Au_{50}	1	50 ±10	3	65
Au_{64}	0.75	64 ±10	2	87

[a] Colloidal gold scatters light (with a yellow Tyndall effect when the granules have a size above 50 nm approximately[25]). However the numbers were calculated from spectrophotometric measurements at the maximum of extinction[1] ($Au_{5\text{ and }17}$, 520 nm; Au_{26}, 525 nm; Au_{32}, 530 nm; Au_{50}, 540 nm; Au_{64}, 545 nm). It was assumed that gold chloride was completely reduced as indicated by Frens[25]. [b] Prepared by reduction of gold chloride with white phosphorus according to Faulk and Taylor[26]. [c] Data are reported with the extreme average dimension measured on 100 granules. [d] Prepared according to Frens[25].

Table 2. Agglutination of human erythrocytes by lectins and lectin-labeled gold granules

Lectin or marker	RBC	n-RBC[a]	ng-RBC[b]	Lectin or marker	RBC	n-RBC
ConA	94,000		188,000	SBA	312,000	
ConA - Au_5	205,500	12,800	51,300	SBA - Au_5	NA	6,400
ConA - Au_{17}	NA,NB[c]	2,600	2,600	SBA - Au_{17}	NA	1,300
ConA - Au_{26}	NA,NB	565	1,130	SBA - Au_{26}	not done	
ConA - Au_{32}	NA,NB	570	1,140	SBA - Au_{32}	570	285
ConA - Au_{50}	NA,NB	1,200	NA	SBA - Au_{50}	NA,NB	150
				SBA - Au_{64}	NA,NB	200
RCA_I	39,000		78,000	WGA	2.08×10^6	
RCA_I - Au_5	205,500	6,400	NA	WGA - Au_5	NA	3,200
RCA_I - Au_{17}	2,600	1,300	5,150	WGA - Au_{17}	5,150	2,600
RCA_I - Au_{26}	565	141	565	WGA - Au_{26}	141	141
RCA_I - Au_{32}	285	71	285	WGA - Au_{32}	71	71
RCA_I - Au_{50}	600	75	NA	WGA - Au_{50}	300	150
RCA_I - Au_{64}	NA,NB	not done	not done	WGA - Au_{64}	400	200

The data indicate the minimal number of lectin molecules or granules per cell causing agglutination in the assay. RBC were prepared from blood (0, Rh^+) freshly drawn into a heparinized syringe. The cells were washed 4 times in saline and suspended to a cell density of 10^8/ml in 0.01 M Tris - 0.15 M NaCl, pH 7.2 containing 0.5 mg/ml Carbowax 20-M (Union Carbide Chemicals Co., New York)[1]. The lectin-labeled gold markers were suspended in the same buffer to an extinction of 2.0 at λ_{max}. Agglutination studies were performed in Takatsy microplates (Cooke Engineering Company, Alexandria, Va). The cells (5×10^6 in 50 µl buffer) were incubated at room temperature with serial dilutions of lectin or lectin-labeled gold marker in U-type plates. Optimal results were obtained when the plates were shaken for 5 sec (Cooke Microshaker) each h for a period of 3 h. Inhibition studies with specific sugars[1–4] indicated that nonspecific adsorption of the markers was practically nil. [a] n-RBC = neuraminidase-treated RBC according to Schnebli et al.[20]. [b] ng-RBC = n-RBC fixed for 15 min in 0.1% glutaraldehyde according to Turner and Liener[27]. [c] NA = no agglutination observed; NB = no binding observed by spectrophotometric measurements and observations by electron microscopy[1,2].

number of binding sites for RCA_I (1.2×10^6) is increased to 1.9×10^6 by neuraminidase[15], as well as that for SBA (1.0×10^6)[19]. In most cases, neuraminidase-treated RBC (n-RBC) were agglutinated by a much smaller number of granules than RBC (table 2). This confirms that cell surface charge is a major factor in RBC agglutination. Since neuraminidase treatment of RBC does not totally remove the negative charge of the cell surface[17,20], the minimal number of granules causing agglutination was still greater for the small probes than for the large ones.

Although Con A-Au_{17-50} is not bound by RBC, n-RBC was agglutinated by Con A-Au_{5-32}. This could indicate that access of Con A to some of its receptors is limited not only by the spacing of the glycoprotein brushes but also by the ionic environment of the receptors[6].

2 sets of evidence contradict increased mobility of the receptors as being an important factor in n-RBC agglutination: a) Microscopical observation indicated that binding of Con A-Au_{17-32} to n-RBC was extremely low (but sufficient to cause agglutination); b) n-RBC were still agglutinable by RCA_I- and Con A-markers after fixation with glutaraldehyde under conditions known to prevent receptor mobility without changing the number of lectin binding sites[21,22] (table 2).

In conclusion, the data in table 2 indicate that no correlation exists between the number of lectin receptors and the minimal number of lectin molecules or lectin-labeled gold granules which causes agglutination of RBC or n-RBC; Con A, SBA and RCA_I receptors are located within brushes of glycoproteins (or clusters of brushes) of increasing spacing, while WGA-receptors are located at the periphery of the brushes; lectin agglutination of RBC is not influenced by the negative charge of the cell surface when the spacer is 26–32 nm in size. Mobility of RBC lectin receptors is not an important factor in agglutination contrary to what is observed with some other cells[23,24]. This new method is under investigation using other cells, untransformed or transformed.

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