Red blood cell targeting to collagen-coated surfaces

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The interaction of human red blood cells carrying antihuman collagen antibody with collagen-coated surfaces was studied. Avidin was used as bifunctional crosslinking agent for the attachment of antibody to the red blood cell surface. Antibody-carrying red blood cells efficiently and specifically bound to collagen-coated surface covering a significant part of the surface. The components of normal blood had an insignificant effect on red blood cell binding. A model of drug targeting to the injured sites(s) of blood vessel wall is proposed.

Drug targeting *Red blood cell* *Antibody attachment* *Collagen* *Avidin*

1. INTRODUCTION

The problem of drug targeting is attracting increasing attention [1–3]. The major principle of targeting consists in attachment of a drug or drug container to a compound able to bind specifically to some part of organ or tissue. Antibodies, hormones and lectins can serve as such compounds. Thus, the drug should be concentrated at the target site and not be evenly distributed over the organism. Advantages of this approach include:

(i) The possibility to reduce side effects of a drug;
(ii) Decrease of the total dose which is especially important when drugs are expensive and not easily available.

At present two systems of targeting are being most intensively developed:

(i) That using 'immunotoxins' [2], which represent the catalytic subunit of natural toxin (e.g., ricin, diphtheria, toxin) attached to an antibody of defined specificity. Immunotoxins are supposed to be mainly used for cancer treatment.
(ii) That using liposomes: Antibodies and other specific proteins are attached (covalently or non-covalently) to the external surface of liposomes [3]. Here, a liposome serves as a 'container' for a drug, and an additional advantage of this system includes insulation of a drug from the surrounding medium; being inside a liposome, a drug does not cause side effects and is not destructed itself.

It is clear that not only liposomes can be used as a container: the use of red blood cells and their ghosts seems rather promising. Red cells of different origin can be 'loaded' by both high molecular mass and low molecular mass compounds and reinjected into bloodstream [4–7] These red blood cells circulate for a longer time (half-time of clearance is equal to several days [4–7]) than liposomes, which can be removed from bloodstream within several hours [8]. It can be assumed that if red blood cells are loaded with a drug, and antibodies are attached to their surface, then such red cells will circulate in the organism delivering their content to the target.

Here, we investigated some properties of our model system; first of all, specificity. We used human red blood cells with anti-human collagen antibody attached to their surface. Collagen-coated plastic surfaces were used as a target. Our system simulated transport of drugs entrapped into red blood cells to a denuded area of blood vessel. This model was chosen [9] as endothelial injury of blood vessel wall with subsequent exposure of subendothelial components, including collagen, could

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be one of the key mechanisms of thrombogenesis, and probably, atherogenesis (review [10–12]). When targeting of biologically active compounds into these areas is mastered it will be possible to interfere with the processes occurring there, and, to some extent, control them.

2. MATERIALS AND METHODS

Blood of healthy volunteers was collected into acid citrate anticoagulant and kept at 4°C. Before use, red blood cells were washed 5 times by centrifugation with phosphate-buffered solution (PBS) (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Type I bovine collagen and rabbit anti-collagen antibody were obtained as in [9]. Type I and III collagens from human placenta and corresponding rabbit antibodies were obtained according to [13]. Type IV and V collagens from human placenta were isolated as in [14]. Avidin was isolated from hen egg white as in [15] (crystallization step omitted). Avidin specific activity determined spectrophotometrically by spectral shift method [16] was equal to 14–15 units/mg. *N*-Hydroxysuccinimide ester of biotin was synthesized according to [17]. Purity of the preparation was tested by thin-layer chromatography on silica-gel (Merck) in chloroform-methanol system (9:1, v/v). Human plasma fibronectin was kindly donated by V.E. Koteliansky.

2.1 *Coating of plastic by collagen*

Collagen solution (1 ml, 20 μg/ml) in 0.02 M bicarbonate buffer (pH 9.6) was added into each well of Falcon Multiwell 3008 and incubated for 2 h at 37°C and overnight at 4°C. Then the well was washed and incubated for 2 h with PBS containing 4 mg bovine serum albumin/ml (Sigma).

2.2. *Attachment of antibodies to red blood cells*

Red blood cells were washed by 5 × 10 ml PBS. Then 0.1 ml 0.1 M borate buffer (pH 9.2) and 3 μl 0.1 M solution of biotin *N*-hydroxysuccinimide ester in dimethylformamide were added into 1 ml red blood cell suspension in PBS (hematocrit value 10%). Reaction mixture was incubated at room temperature for 20 min, then red cells were washed by centrifugation by 5 × 10 ml PBS. 1 ml suspension of modified red blood cells (hematocrit value 10%) was added to 1 ml avidin solution, 1 mg/ml in PBS, and incubated with agitation for 1 h at 4°C. Red blood cells were washed off non-bound avidin by 3 × 10 ml PBS.

Biotin *N*-hydroxysuccinimide (0.1 M) in dimethylformamide was added to 0.2–1.0 ml antibody or rabbit non-immune IgG in PBS (0.3–1.0 mg/ml) to 0.3 mM final conc. and incubated for 1 h at 4°C. The reaction mixture was dialyzed overnight at 4°C against 5 l PBS. Modified protein solution (15 μl) was added to 1 ml suspension of red blood cells coated by avidin (hematocrit value 10%), and incubated for 1 h with agitation at 4°C. Red blood cells were washed off non-bound protein by centrifugation by 3 × 10 ml PBS. In experiments on quantitative determination of protein binding to the red blood cells ^{125}I-labelled rabbit IgG was added into initial protein solution as tracer.

2.3. *Incubation of red blood cells in collagen-coated wells*

Red blood cell suspension (250 μl, 4×10^7 cells/ml) was added into each collagen-coated well and incubated for 1 h at room temperature with occasional strirring or permanent rotation around a horizontal axis (Fisher Roto-Rack, Model 343, 15 rev./min). Then wells were washed with 4 × 1 ml PBS with rotation (for 5 min each time). Bound red blood cells were fixed by 1 ml 0.5% glutaraldehyde in PBS overnight at 4°C and washed with water. The amount of bound red blood cells was determined by microscopic counting in 6C-2 profile projector (Nikon) with 400 × objective.

3. RESULTS AND DISCUSSION

For attachment of antibodies to red blood cell surface we used avidin–biotin non-covalent interaction. Avidin has 4 biotin-binding sites and can serve as bifunctional cross-linking agent for molecules carrying biotin groups [18,19]. The scheme of antibody attachment is shown in fig. 1. At first, red blood cells and antibody were separately treated by biotin *N*-hydroxysuccinimide ester which resulted in the appearance of biotin groups on red blood cell surface and in antibody molecule. By titration with trinitrobenzene sulfonic acid [21] it was found that this treatment

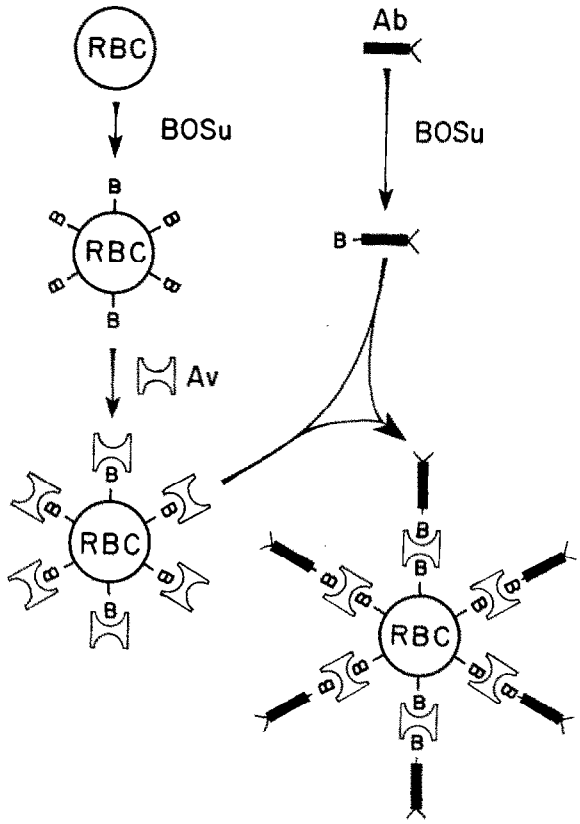

Fig. 1. Scheme of antibody attachment to red blood cell surface. *Abbreviations:* RBC, red blood cells; B, biotin; BOSu, biotin *N*-hydroxysuccinimide ester; Av, avidin; Ab, antibody.

led to modification of 15% amino groups of antibody molecule. According to the data obtained by enzyme-linked immunosorbent assay this modification does not decrease antibody activity. Red blood cells were then incubated in avidin solution, non-bound avidin was removed and biotin-modified antibody was added. As a result, antibodies attached to the red blood cell surface through avidin molecule. Besides, in this case avidin serves as a spacer arm 55 Å in length [19]. Since avidin–biotin interaction is non-covalent, it is very rapid ($k_{ass} = 7 \times 10^7\ M^{-1}.s^{-1}$), and attachment does not require prolonged incubations. This interaction is also very strong ($K_d = 1.3 \times 10^{-15}$ M [19]) and the half-time avidin–biotin complex dissociation is 200 days [19]. Due to these peculiarities the method of attachment is very efficient. For example, adding only 5 μg biotin-modified antibody to 2×10^8 red blood cells it is possible to attach 30% of added antibody within 1 h, which is practically impossible for methods of covalent attachment. In this case 30 000 antibody molecules attach/red blood cell. In a typical experiment 100 μg antibody were usually added per 10^9 red blood cells providing 80 000–100 000 antibody molecules attached per red blood cell with an attachment efficiency of ~25%.

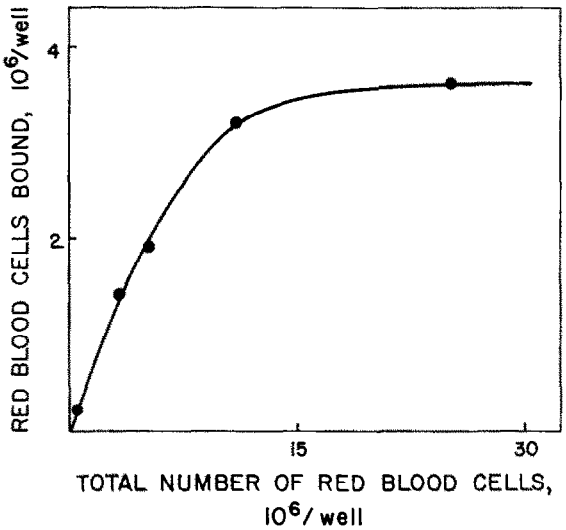

Fig. 2. Dependence of red blood cell binding on their concentration. Red blood cells carrying antibody were incubated in wells coated with type I human collagen in section 2.

Incubation of antibody-carrying red blood cells in collagen-coated wells led to the binding of erythrocytes to the collagen-coated surface. This binding depends on red blood cell concentration and reaches 3.6×10^6 red blood cells/well with a surface area of 2 cm^2 (fig. 2)*. Binding of red blood cells carrying non-immune IgG is equal to $4–8 \times 10^3$ cells/well (not shown in fig. 2 due to a discrepancy of scale). Thus, attachment of antibody to red blood cells instead of non-immune IgG leads to the increase of red blood cells binding to collagen by 400–800 times. The same results were obtained when type I bovine collagen and corresponding rabbit antibodies were used (not shown).

* These results were obtained using collagen absorbed at the well surface. Similar results were obtained with the wells coated by fibrillar collagen, as in [9].

From fig. 2 one can calculate that at maximal binding the surface area occupied by one red blood cell is $\simeq 70\,\mu m^2$. Since human red blood cell represents a disc of 8 μm diam., the area occupied by a flatly lying red blood cell is $\simeq 50\,\mu m^2$. Thus, in our experiments bound cells occupy ~70% of available surface.

Specificity of red blood cell binding to collagen was also tested in experiments with blocking of collagen antigenic determinants. For this purpose, collagen in wells was preincubated with different amounts of anti-collagen antibody. As the amount of antibody added into a well increases, red blood cell binding decreases (fig. 3); at $>10\,\mu g$ antibody/well, binding is practically absent (fig. 3). These results confirm that red blood cell binding strictly depends on the interaction of antibody with collagen antigenic determinants.

Specificity of red blood cell binding according to the type of protein attached to the plastic surface

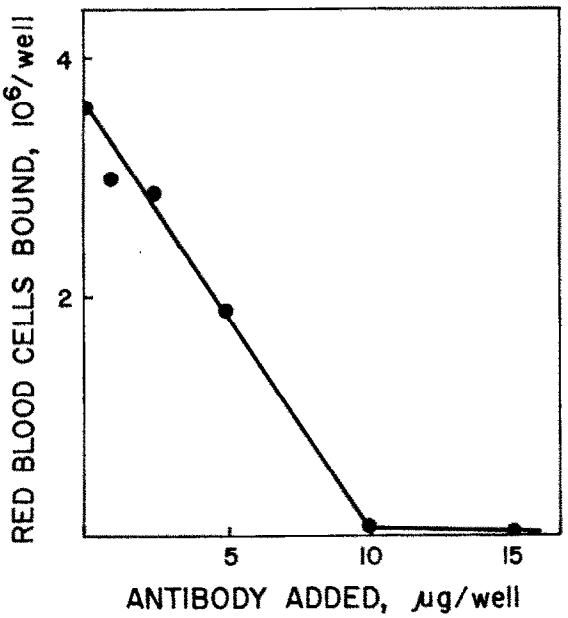

Fig. 3. Effect of collagen preincubation with antibody on red cell binding. In wells coated with type I human collagen the indicated amounts of antibody to this collagen were added in 0.5 ml PBS containing 4 mg/ml bovine serum albumin and incubated for 1 h at room temperature.Then the wells were washed by 3 × 1 ml PBS with bovine serum albumin. Red blood cell incubation, fixation and counting of bound red cells were performed as in section 2.

Table 1

Binding of antibody-carrying red blood cells to the surfaces coated with different types of collagen

Collagen type	Red blood cells bound (10^6/well)
I	3.4
III	<0.01
IV	<0.01
V	<0.01

Wells were coated with human collagen of different types as in section 2. PBS (250 μl) containing 10^7 red blood cells carrying antibody to type I human collagen were added into each well. Incubation, fixation and counting of bound red cells were performed as in section 2. Binding of red blood cells carrying non-immune globulin was $\leqslant 10^4$ cells/well in all cases.

was tested by coating wells with human collagen of different types. Table 1 demonstrates that binding of red blood cells carrying antibody to type I collagen occurs only on type I collagen. Binding is practically absent with collagens of types III–V.

We also tested the effect of some components of human normal blood on red blood cell binding to collagen. For instance, such collagen-binding protein as fibronectin did not affect the binding up to its physiological concentrations; i.e., up to 0.3 mg/ml [20]. This can be accounted for by the suggestion that fibronectin-binding sites of collagen are insignificantly overlapped with most of its antigenic determinants. Other blood proteins also did not affect red blood cell interaction with collagen, since addition of human blood plasma to $\leqslant$ 50% (v/v) did not change the binding (not shown).

To study the influence of cellular blood components on red blood cell binding we added different amounts of intact erythrocytes into incubation mixture. This addition led to the inhibition of antibody-carrying red blood cell binding (fig. 4). At concentrations of intact erythrocytes corresponding to physiological hematocrit values (50%), inhibition was 60%. Thus, presence of > 100-fold excess of intact red blood cells decreased binding by 2.5-fold. Evidently, inhibition is due to the blocking of the available surface and, most probably, it can be overcome by increase of incubation time and intensity of agitation. This is confirmed by the fact that if continuous agitation

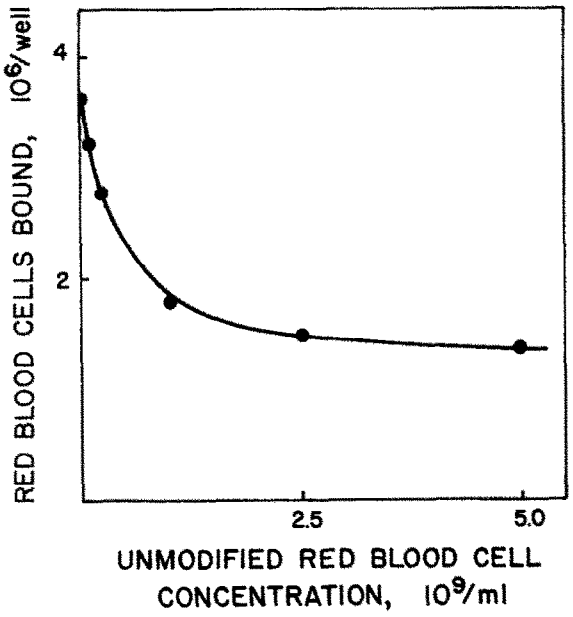

Fig. 4. Effect of intact red blood cells on the binding of antibody-carrying red blood cells. 10^7 red blood cells carrying antibodies to type I collagen and different amounts of intact red blood cells were added into each well coated with human collagen. The total volume in the well is 250 μl. Incubation, fixation and counting of bound red blood cells were performed as in section 2.

is replaced by occasional agitation, the degree of inhibition increases up to 90% at 5×10^9 intact red blood cells/ml.

We have demonstrated the possibility of targeting of red blood cells carrying antibody to surfaces coated with antigen, particularly with human collagen. In this case, red blood cell binding is highly effective and specific, and the effect of blood components is either absent or expressed rather weakly. It is not possible to apply the results obtained directly to the case of denuded vessel in vivo:

(i) Collagen of blood vessel wall can be linked with other structural components (e.g., proteins, proteoglycans), which can mask its antigenic determinants;

(ii) Unevenness of microrelief of denuded vessel wall can be comparable to erythrocyte size which will sterically prevent the contact of antibodies with collagen at red blood cell surface;

(iii) We did not study the effect of platelets which very actively interact with collagen.

All these questions should be tested experimentally on the denuded vessel.

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