Brief Definitive Report

CONCANAVALIN A-MEDIATED BINDING AND SPHERING OF HUMAN RED BLOOD CELLS BY HOMOLOGOUS MONOCYTES*

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Red blood cells (RBCs) sensitized with immunoglobulin G (IgG) or with the third component of complement (C3) are bound to the surface of mononuclear phagocytes (1, 2). This cell-cell interaction results in damage to the RBC, characterized in vitro by enhanced RBC osmotic fragility and spherocytosis (1, 3). A similar process in vivo appears to underlie the spherocytosis characteristic of warm antibody (IgG)-induced hemolysis (1) and found in some patients with cold agglutinin (IgM-C3) disease (4).

The multivalent plant lectin concanavalin A (Con A) binds to the surface membrane of many cells and may mediate the association of like cells (as in RBC agglutination) or that of dissimilar cells. More particularly, Con A has been used to bind monocytes or macrophages to several target cells, including bacteria (5), murine tumor cells (6), and murine RBCs (7). The present study explores the binding by human peripheral blood monocytes of homologous RBCs coated with Con A and the consequent damage inflicted on the bound RBC.

Materials and Methods

Mononuclear Cell Monolayers. Mononuclear cell monolayers were prepared from density gradient centrifugation of human peripheral blood as previously described (8).

Concanavalin A. Con A ("Grade IV"; Sigma Chemical Co., St. Louis, Mo.) was labeled with ^{125}I using the method of McConahey and Dixon (9). The labeled protein had identical biologic activity to unlabeled Con A (by hemagglutination titer and monocyte binding, see below). α-Methyl-D-mannopyranoside (α-MM) (Sigma Chemical Co.) was used to inhibit the saccharide-binding sites of Con A.

Sensitization of RBCs. Heparinized whole blood was washed twice with 0.01 M EDTA in isotonic veronal-buffered saline (pH 7.4) containing 0.1% gelatin. RBCs (1×10^8 RBC/ml in Hanks' balanced salt solution [HBSS]) were sensitized at 37°C for 30 min with an equal volume of Con A in 0.15 M NaCl, washed once, and resuspended to 5×10^7/ml in HBSS. To determine the number of molecules of Con A per cell, RBCs were sensitized with ^{125}I-radiolabeled Con A, centrifuged at 1,000 *g*, washed, resuspended in buffer, and their radioactivity determined in a gamma counter (Searle Analytic, Inc., Des Plaines, Ill.). Con A was assumed to be largely tetrameric with a mol wt of 112,000 daltons under the conditions of the binding experiments (10). IgG-sensitized RBCs were prepared as previously described (8) using human anti-D antiserum (Sera-Tec Biologicals,

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New Brunswick, N. J.). The average number of IgG molecules per RBC was determined by C1 fixation and transfer (11).

Monocyte Binding and Phagocytosis of RBCs. To examine RBC binding by monocytes, 1 ml of HBSS containing 5×10^7 RBCs was added to tissue culture dishes containing the monolayer. After incubation at 37°C, dishes were washed, air dried, and stained with Wright's-Giemsa. Blinded replicate plates were examined by light microscopy. The number of RBCs attached to 100 monocytes was recorded and the data expressed as mean RBCs per monocyte and percentage of monocytes binding ≥ 2 RBCs. Phagocytosis of RBCs was examined after hypotonic lysis of extracellular RBCs and expressed as both the mean number of RBCs within 100 monocytes and the mean percentage of monocytes containing any RBCs.

Determination of RBC Osmotic Fragility. ^{51}Cr sodium dichromate (100 μCi/ml of whole blood) labeled RBCs were sensitized with Con A and allowed to bind to monocytes. Unbound RBCs were washed away and 4 ml of phosphate-buffered saline, pH 7.4, of varying tonicity was added to each of four replicate plates. After 10 min at room temperature 2.5 ml was removed for determination of radioactivity. To determine the osmotic fragility of RBCs in suspension, cells were washed, resuspended to 1×10^8 cells/ml, and 20 μl were added to 4 ml of osmotic fragility solution. After centrifugation, supernatant radioactivity was counted and percent hemolysis determined by comparison to the amount of radioactivity at 0.1 grams of sodium chloride/100 ml.

Results

Sensitization of Red Cells. RBCs (2.5×10^7/ml) were incubated at 37°C for 30 min in the presence of ^{125}I-labeled Con A. This resulted in 12% (range = 7.3–15.1%) of the labeled material becoming cell associated. The percent Con A bound remained constant over the range of Con A concentrations from 0.45 to 45 μg/ml. A mean 24.4% (range 17.1–28.1%) of the Con A which was bound to the RBC surface eluted over the course of 2 h. Subsequent vigorous washing removed only 6.7% (range = 5.4–9.0%) of the remaining labeled Con A. Thus, the mean amount of Con A bound to RBCs during their 2-h incubation with monocytes was equivalent to approximately 10% of that present in the medium at the time of RBC sensitization.

Monocyte-Red Cell Interaction: The Rosette. Incubation of Con A-coated RBCs with monocytes resulted in binding to form a rosette (Fig. 1). Both the number of RBCs bound per monocyte and the percent of monocytes that bound two or more RBCs (Table I) depended on the amount of Con A on the RBC surface. At a sensitizing concentration of 10 μg/ml (calculated from ^{125}I-Con A data to represent approximately 10^5 molecules of tetrameric Con A per RBC) a mean of 2.2 RBCs was bound per monocyte and approximately 50% of monocytes bound at least two RBCs.

The kinetics of RBC-monocyte binding in HBSS was investigated in three experiments using sensitizing concentrations of Con A of 42 μg/ml. RBC binding was maximal at 90 min and decreased thereafter. By 15 min nearly 90% of monocytes had bound two or more RBCs, and the percentage subsequently rose to 95%.

Monocytes never bound unsensitized RBCs. Even after pretreatment of the monocyte monolayer with Con A in concentrations ranging from 6.25 to 1,000 μg/ml and incubation at various temperatures (4°C, 22°C, and 37°C), unsensitized RBCs were not bound.

When Con A-sensitized RBCs were incubated with monocytes in the presence of 0.1 M α-MM, rosette formation was totally inhibited. To test the ability of α-MM to remove bound RBCs, rosettes were formed for 90 min, washed, and at 0,

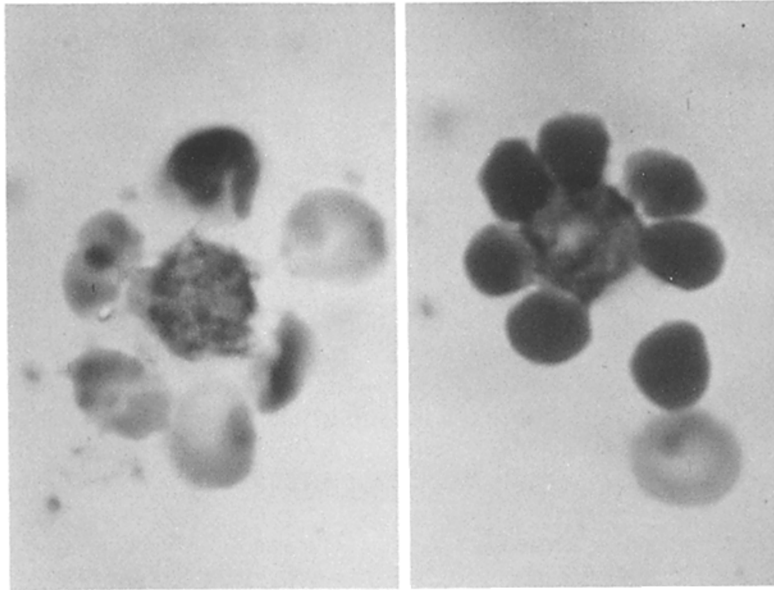

FIG. 1. Con A-sensitized RBC-monocyte rosettes. The left panel depicts a rosette after 1 h incubation. RBCs are distorted but not sphered. In the right panel is a rosette after 4 h incubation. Bound RBCs are spherocytic. The unbound cell (lower right) is morphologically normal.

TABLE I
Dose-Response: Concentration of Con A and Binding of RBCs to Monocytes

Sensitizing concentration of Con A*	RBCs bound/monocyte‡	% Monocytes binding‡ ≥2 RBCs
μg/ml		
8	0.8 (0.1–1.4)	22 (2–42)
10	2.2 (2.0–2.5)	56 (49–62)
16	3.2 (2.4–4.0)	75 (57–90)
21	4.2 (3.3–5.4)	88 (79–94)
32	4.8 (4.5–5.0)	88 (87–89)
42	5.3 (4.9–6.4)	93 (87–97)
64	6.8 (6.5–7.1)	96 (95–97)
84	7.0 (5.8–8.1)	97 (94–99)

* Sensitized RBCs were incubated with monocytes in HBSS for 90 min.
‡ Mean and range of four replicate plates for two to five experiments.

1.5, and 2.5 h α-MM was added for 0.5 h. This resulted in the release of 100, 95, and 81%, respectively, of bound RBCs. Pretreatment of the monolayer with α-MM followed by washing did not alter monocyte binding of Con A-sensitized RBCs.

Interaction of Monocyte IgG and Con A "Receptors." Neither pretreatment of monocytes with purified myeloma-derived IgG nor the addition of IgG concomitant with the addition of Con A-sensitized RBCs had any effect on the binding of Con A-sensitized RBCs. In each case binding of IgG anti-D-sensitized RBCs was blocked. Pretreatment of the monolayer with Con A at a concentration of 42 μg/ml decreased the binding of RBCs sensitized with Con A. In

contrast, pretreatment of monocyte monolayers with Con A increased, by a mean of 58%, the binding of IgG-sensitized RBCs. The facilitation of binding of IgG-coated RBCs was more marked when either the number of IgG molecules (200–1,000) bound per RBC or the concentration of Con A to which the monocyte was exposed was increased. Facilitation also occurred with RBCs sensitized with IgG anti-D isolated by Sephadex G-200 chromatography to be free of detectable IgM. The facilitation of IgG-RBC binding by Con A was blocked by the prior addition of α-MM and reversed by addition of α-MM after binding of IgG sensitized RBCs.

Osmotic Fragility and Sphering of Red Cells Bound to Monocytes. By 4 h of incubation ^{51}Cr-labeled RBCs bound to monocytes were significantly osmotically fragile compared to those incubated in the absence of monocytes (Fig. 2). RBCs removed from the monocyte with α-MM after 4 h of incubation were also osmotically fragile as compared to cells that had not become monocyte adherent. By light microscopy, RBC attached to monocytes for 1 h appeared distorted, but often maintained an area of central pallor, whereas RBCs attached for 4 h were spherocytic (Fig. 1).

Phagocytosis of Con A-Sensitized RBC. In three of three experiments, the percentage of monocytes ingesting RBCs never exceeded 6.5% and the mean was 4% at 1 h and 2% at 4 h. The total number of RBCs interiorized was also small, with a mean of 4.5 RBCs per 100 monocytes at 1 h and 2.0 RBCs per 100 monocytes at 4 h.

Discussion

The recognition (binding) of a variety of objects, from latex spheres to nucleated cells, is a fundamental property of the phagocyte. Binding may be of no apparent consequence, as in macrophage interaction with mycoplasma organisms (12) or RBCs sensitized with only $F(ab')_2$ immunoglobulin fragments (13). Under the appropriate circumstances, however, binding is the prelude to ingestion of the object and its subsequent attack by digestive enzymes. Intermediate between the poles of inconsequential binding and "phagocytic recognition" may be those phenomena in which binding leads to an alteration of the bound object without it being ingested. The sphering of RBCs bound to monocytes with Con A, as in the present study, or with IgG anti-D (1) or C3 (3) is one manifestation of this process. Con A-mediated sphering may be the counterpart of the old observation of Ham and Castle that injection of Con A into dogs produces in vivo spherocytosis and hemolysis (14).

Several lines of evidence implicate Con A as directly linking RBC and monocyte. First, unsensitized RBCs were not bound. Second, the degree of binding of sensitized RBCs was dependent on the number of molecules of Con A placed on the RBC surface. Third, binding was both prevented and reversed by α-MM, Con A's specific saccharide inhibitor. Finally, Con A placed on the monocyte inhibited the subsequent attachment of Con A-coated RBCs.

While Con A-mediated binding and sphering of RBCs parallels immune recognition and damage, it does not appear to involve the IgG receptor. Nevertheless, a functional interaction between the IgG and Con A receptors could be demonstrated, since binding of IgG-sensitized RBCs was increased by pretreating monocytes with Con A. Possibly once a recognition point is established

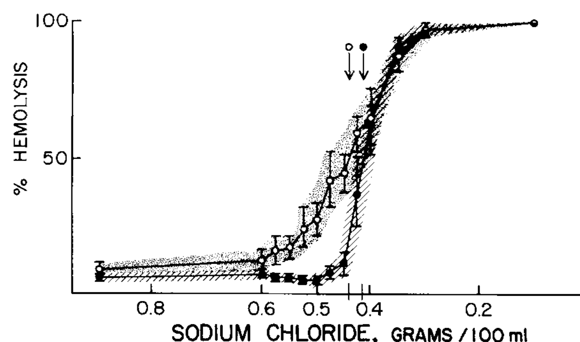

FIG. 2. Osmotic fragility curves of RBCs bound to monocytes for 4 h (open circles) or incubated without monocytes for 4 h (closed circles). The arrows designate the concentration of sodium chloride at which 50% of ^{51}Cr-labeled Con A-sensitized RBCs were lysed. The data represent means ± SEM for quadruplicate plates in six of six experiments.

physical association of RBC and phagocyte occurs and binding becomes established if other molecules of either IgG or Con A engage their complementary monocyte receptors. Enhanced binding did not occur because of Con A binding to saccharide residues on contaminating IgM as the same phenomenon was seen with column purified IgG. It is possible that the small amount of carbohydrate associated with IgG anti-D (15) is the site of attachment of Con A. That enhanced binding is probably not mediated by a change in the state of "activation" (16) of the mononuclear phagocyte is indicated by the reversal of enhancement by α-MM.

The precise nature of the events involved in the transition from recognition to phagocytosis are as yet only incompletely understood. In our system, phagocytosis of Con A-sensitized RBCs was minimal, as it is under certain circumstances with IgG coating the RBC (1) and under many circumstances in which C3 is the only substance sensitizing the RBC (2, 17, 18). Despite minimal interiorization of Con A-coated RBCs, spherocytes were produced at the phagocytic cell surface. That sphering can occur without bulk phagocytosis and that phagocytosis can proceed without producing spherocytes (7) suggests important functional differences in the two phenomena. While rosetting may be "a truncated version of ingestion" (19), it provides a means by which surface-bound particles can be altered – the RBC sphered, the antigen processed, and the lymphocyte communicated with – without being engulfed and destroyed.

Summary

Human red blood cells sensitized with concanavalin A became bound to homologous peripheral blood monocytes. Binding occurred at a concentration of 10^5 molecules of tetrameric Con A per red blood cell (RBC) and increased with additional Con A. RBC binding began within 5 min and was maximal at 90 min. Phagocytosis of sensitized RBCs was minimal. RBC attachment was prevented by 0.01 M α-methyl-D-mannopyranoside, and, once the RBC-monocyte rosette was established, bound RBCs were largely removed with this specific saccharide inhibitor of Con A. RBCs attached to monocytes became spherocytic and osmotically fragile. The recognition of concanavalin A (Con A)-coated RBCs was not mediated through the monocyte IgG-Fc receptor. These studies demonstrate

that, like IgG and C3b, Con A is capable of mediating the binding of human RBCs to human monocytes. Red cells so bound are damaged at the monocyte surface.

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