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Attachment of Modified Erythrocytes to Phagocytic Cells in Absence of Serum.* (31749)

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Phagocytosis by macrophages *in vitro* requires in most cases the presence of serum in the medium or pretreatment of the particulates with serum or serum fractions(1,2,3). The overall phagocytic process can be shown in some instances to involve discrete attachment and ingestion steps(3,4). The first step embodies the "recognition" of "foreign" particulates or of endogenous aged or damaged body constituents, while "self" components are spared(5). Because of the large amount of prior knowledge concerning erythrocytes as well as their size and availability, these particles were chosen for the study of this early phagocyte-particle interaction. It was found that erythrocytes modified by various procedures attached to macrophages, but not to granulocytes, in the absence of added serum factors. This interaction was temperature dependent and was blocked by prior trypsinization of the macrophages. Preliminary communications of this work have appeared previously(6,7).

Materials and methods. Macrophages were obtained by washing the peritoneal cavity of NCS mice (Rockefeller University colony) with 4 ml buffered saline (9 vol 0.85% NaCl solution plus 1 vol M/15 potassium phosphates pH 7.3), and permitted to attach to coverglasses(8). Mice of both sexes weighing 20-25 g were used.

Horse peripheral blood leucocytes (70-80% polymorphs) were obtained after sedimentation of the erythrocytes and centrifugation of the leucocyte-rich plasma. Heparin or citrate were used as anticoagulants. The resulting leucocyte pellets were washed twice and resuspended in buffered saline before attachment to coverglasses.

Sheep red cells were obtained from the Animal Blood Centre, Syracuse, N. Y. Mouse, horse, rabbit and human cells were collected from heparinized blood. Erythrocytes were washed 4-6 times in buffered saline before and after the following treatments. Each of the agents was dissolved in buffered saline pH 7.3. Except when otherwise noted the conditions given apply to mouse erythrocytes at 22°C. *Glutaraldehyde:* 10 volumes of 1-5% glutaraldehyde were added to red cells and incubated for 24-48 hours at 5°C. Red cells obtained from the horse, mouse, rabbit and human were processed in the same fashion. These cells were stable at 5°C for at least 2 months. *Tannic acid*(9) was used at 25 μg/ml for 20 minutes. *Periodate*(10) M/500 was used at pH 6.1 for 10 minutes in the dark. *Polylysine*(11) obtained from Yeda, Rehovot, Israel, was applied at 5-10 μg/ml for 10 minutes. *Colloidal silica* ("Syton 200," lot no. 5039, obtained from Mon-

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santo Chemical, St. Louis, Mo.) was made up to .05% v/v and added to horse cells from 45 minutes(12). *Heat treatment:* red cell suspensions were rapidly injected with agitation in buffered saline at 90°C. After 2 minutes the suspension was allowed to cool to room temperature. Red cell fragments and aggregates were eliminated by two 4-minute centrifugation cycles at 40 and 350 *g*. *Trypsin* at 400 μg/ml was applied for 40 minutes at 37°C. n-*Ethylmaleimide* was used at 2×10^{-3} M for one hour. *Sensitization with antibody:* sheep red cells were treated with 1/800 to 1/3200 of anti-sheep hemolysin (Baltimore Biological Laboratories) for 20 minutes at 34°C. The sensitized erythrocytes were included for comparative purposes.

After rinsing the phagocytes with buffered saline, the erythrocyte suspensions (10 to 30,000 per μl) were applied for 15 minutes at 37°C, except for the antibody-coated red cells which were overlaid at room temperature for one hour. The preparations were washed by agitation for 2-4 seconds in 50 ml volumes of buffered saline, fixed in 1% glutaraldehyde and stained with Giemsa. Adherence of erythrocytes to the macrophage surface was scored microscopically. At "high" attachment 75% or more of the macrophages had 2-10 red cells adherent to them, forming a rosette. In what is described as "low" attachment, less than 25% of the macrophages had attached red cells. In the latter case fewer red cells attached per macrophage. For the study of temperature dependence, attachment was observed on samples of macrophages and treated red cells incubated at 2°, 22° and 37°C. In the trypsinization experiments, the macrophages, before red cell attachment, were treated with 250-500 μg/ml of the crystalline enzyme in Hanks' solution for 30-60 minutes at either 37° or 2°C. Controls were pre-incubated in Hanks' solution alone.

Results. Homologous or heterologous erythrocytes, as well as trypsinized and n-ethylmaleimide treated cells did not attach to macrophages in the serum-free medium employed. In contrast, red cells modified by exposure to glutaraldehyde, tannnic acid (Fig. 1), periodate, polylysine, colloidal silica or heat (Fig. 2), exhibited typical rosette attachment to the macrophage surface, which appeared similar to the attachment exhibited by red cells sensitized with specific antibody. Aldehyde or heat treated red cells attached to macrophages but not to polymorphs, whereas after exposure to tannic acid, periodate, polylysine, colloidal silica or specific antibody, the erythrocytes occasionally also adhered to polymorphs, lymphocytes and mast cells. Attachment of the red cells to glass was in every instance minimal or absent. Trypsinization of erythrocytes prior to their exposure to glutaraldehyde or periodate did not affect their subsequent tendency to attach to macrophages.

TABLE I. Attachment of Modified Erythrocytes to Macrophages. Effect of temperature and effect of trypsinization of the macrophages.

Red cell treatment	Red cell species tested	Temperature dependence of attachment	Attachment after trypsinization of the macrophages
Glutaraldehyde	M, H, Hu, R	+	abolished
Tannic acid	M	+	reduced
Periodate	M	not done	not done
Polylysine	M	" "	" "
Colloidal silica	H	+	reduced
Heat	M	+	abolished
Specific antibody	S	++	reduced

M = mouse; H = horse; Hu = human; R = rabbit; S = sheep red cells.
+, attachment 'low' at 2°C, intermediate at 22°C, 'high' at 37°C.
++, attachment 'low' at 2°C, 'high' at 22°C.

In all instances studied, attachment of the treated red cells to macrophages was temperature dependent, as shown in Table I. Attachment was low at 2°C, intermediate at 22°C and maximal at 37°C, except for antibody-coated cells, the attachment of which was maximal at 22°C. Preincubation of macrophages at 2°C did not influence the subsequent attachment of aldehyde-treated erythrocytes at 37°C.

Preincubation of the macrophages at 37°C with crystalline trypsin abolished the subsequent adherence to these cells of aldehyde- or heat-treated erythrocytes, and reduced the

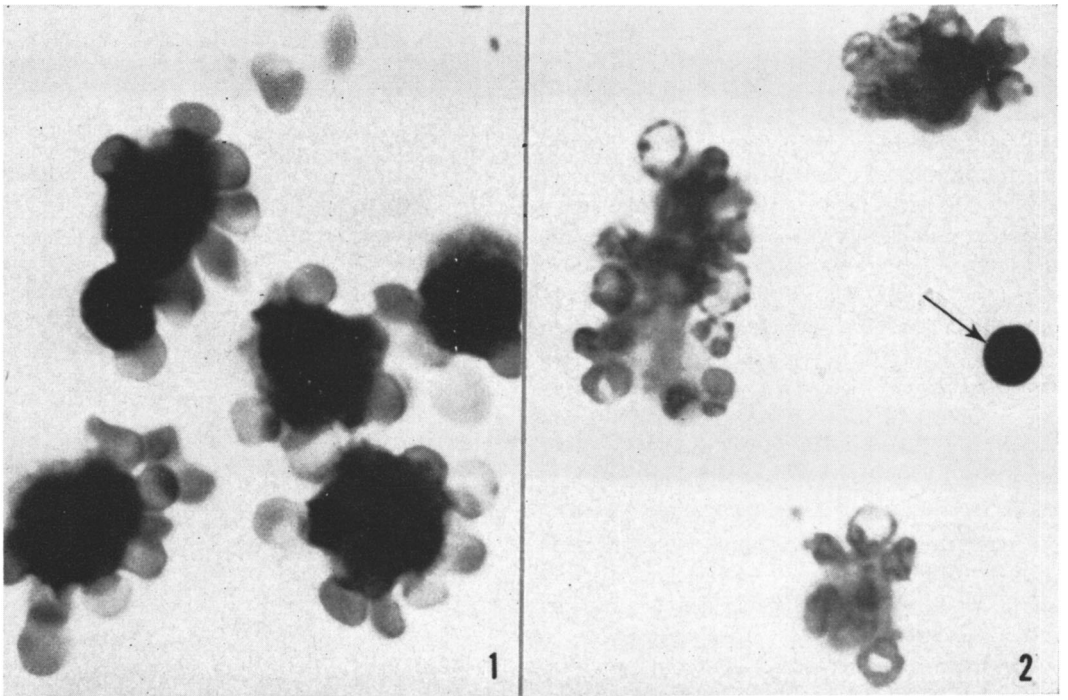

FIG. 1 and 2. (× 900). Mouse peritoneal macrophages after attachment of tannic acid (Fig. 1) or heart treated (Fig. 2) red blood cells. The attachment, in both cases is classified as "high." In Fig. 2, lymphocyte (arrow) does not show erythrocyte adherence.

adherence of tannic acid, colloidal silica or antibody treated cells (Table I). The trypsinization of macrophages at 2°C did not similarly alter their ability to interact with modified erythrocytes.

Discussion. We have shown that the modification of red cells by a variety of means leads to their attachment to macrophages in saline medium, in a manner apparently similar to the attachment of sensitized erythrocytes previously described(3,4). In the early literature(1), treatment of particulates with certain chemicals, termed "artificial opsonins," including tannin and some trivalent cations, was reported to induce phagocytosis in the absence of serum. With one exception(13), no evidence for a discrete attachment step promoted by such treatments was provided. Adherence of effete red cells to macrophages has also been described(14).

The different behavior of polymorphonuclear leucocytes and of macrophages in this interaction with modified red blood cells indicates that these cell types differ in the intimate chemical nature of their surfaces or in their requirements for binding to various particulates.

The diminished attachment of modified red cells to macrophages at 2°C as compared to 37°C might indicate that the interaction is itself temperature dependent, or that it is indirectly dependent on macrophage metabolism, through, for example, the synthesis or excretion of a surface factor. The role of a surface protein is suggested by the fact that preincubation of the macrophages with trypsin reduces or abolishes subsequent attachment of modified red cells. That trypsin is indeed acting enzymatically is indicated by its ineffectiveness at 2°C. Trypsinization of macrophages was previously reported to inhibit effete red cell attachment(14).

Attachment of red cells after procedures as diverse as those used in these studies might involve different mechanisms. One possibility is that the interaction is mediated through "natural" antibodies carried by the macrophages (*cf.* ref. 2,3). The existence in human serum of agglutinins against autologous, periodate treated erythrocytes(15) lends

support to this mechanism. Alternatively, red cell treatments effective in promoting attachment might impart to the erythrocyte surface molecular configurations that mimic those of antigen bound antibody. It should be pointed out that several of the treatments used, namely heat, aldehyde, tannic acid and polylysine, induce conformational changes in proteins, and that macrophages display marked affinity, both *in vivo*(16) and *in vitro* (17) for denatured proteins. That a change in the red cell surface proteins might underlie the described interaction is indicated by the attachment to macrophages of aldehyde treated ovalbumin crystals and gelatin DNA coacervates(18) in saline medium (unpublished results).

Summary. Homologous or heterologous red cells subjected to a number of treatments attached to mouse peritoneal macrophages in a saline, protein free medium in contrast to the lack of attachment of untreated red cells. Effective treatments included heat, glutaraldehyde, tannic acid, periodate, polylysine and colloidal silica. Under the same conditions, the modified erythrocytes showed little or no attachment to polymorphonuclear leucocytes. Trypsin or n-ethylmaleimide treatment of erythrocytes did not promote attachment to either cell type. Attachment of treated red cells was temperature dependent, and was abolished or reduced by preincubation of the macrophages with trypsin.

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Plasma Histaminase Activity in Various Mammalian Species; A Rapid Method of Assay.* (31750)

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While investigating the effects of administered histamine and compound 48/80 on plasma histamine levels in monkeys, we observed that squirrel monkeys (*Saimiri sciureus*) exhibited elevations of plasma histamine shortly after administration whereas rhesus monkeys (*Macaca mulatta*) did not. But both species demonstrated signs of increased circulatory histamine as evidenced by gross observations (erythema, hypopnea, reduced blood pressure).† Apparently, rhesus monkeys are unique among mammalian species including

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