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Erythrocyte enhancement of C3b-mediated phagocytosis by human neutrophils *in vitro*: a combined effect of the erythrocyte complement receptors CR1 and erythrocyte scavengers to reactive oxygen metabolites (ROM)

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Summary. Human erythrocyte CR1 receptors have been shown to bind complement-fixing immune complexes and, thus, facilitate their elimination from the circulation. The autotoxic effect of free radicals released from phagocytes during phagocytosis can be alleviated by scavengers like catalase and superoxide dismutase. Erythrocytes are known to contain these antioxidants.

This study showed that 74% of opsonized yeast particles adhered to human erythrocytes. No difference was seen between yeast opsonized with C3b and yeast opsonized with both IgG and C3b. This adherence was due to the C3b receptor (CR1), as monoclonal antibodies against the CR1 receptor could abrogate the adherence. The yeast phagocytosis by neutrophils was increased by 15% when yeast-C3b was used, and by 34% when yeast-IgG/C3b was used in the presence of human red blood cells. The increase of phagocytosis was not seen when rat erythrocytes (lacking CR1) were present. The cytochrome *c* reduction decreased with the presence of human erythrocytes during phagocytosis, indicating a scavenging effect on the superoxide anions. The addition of scavengers or erythrocyte lysate, instead of erythrocytes, enhanced phagocytosis of yeast-IgG/C3b to at least the same extent as the erythrocytes.

These observations suggest that human erythrocytes primarily enhance phagocytosis through the scavenging effect of those erythrocytes which are concurrently attached with the prey through its CR1 receptor, and then attached to the PMN.

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INTRODUCTION

Primate erythrocytes are known to possess receptors for complement factor C3b (CR1) (Hajos *et al.*, 1978; Fearon, 1984), but the biological role of these receptors is not fully understood. The capability of the erythrocytes to bind complement-fixing immune complexes (IC) (Fearon, 1980) has been proposed to be of importance in the removal of IC from the circulation to the liver and spleen (Cornacoff *et al.*, 1983; Medof *et al.*, 1983a). The CR1 receptor has also been proposed as important in degrading large IC to smaller ones (Medof & Prince, 1983b; Medof *et al.*, 1982a), and in the regulation of the fluid phase activation of the complement system due to its β-1H activity (Medof *et al.*, 1983a; Iida & Nussenzweig, 1981). Besides possessing CR1 receptors, erythrocytes contain scavengers of reactive oxygen metabolites (ROM) (Lynch & Fridovich, 1978) which can participate in the phagocytic process (Hand & King-Thompson, 1983). We have recently shown that ROM-scavengers enhance opsonin-mediated phagocytosis (Stendahl *et al.*, 1984) in aggreement with the results of others showing an

autotoxic effect of ROM on neutrophils (Roos *et al.*, 1980) or a receptor-modulating effect on macrophages (Thaw *et al.*, 1984). In this study, it has been possible to investigate the role of erythrocyte anti-oxidants on the phagocytic process by using targets with differing abilities to induce a release of ROM from neutrophils (Hed & Stendahl, 1982; Hed, Stendahl & Sundquist, 1983), and by using erythrocytes with (human) and without (rat) CR1 receptors (Hajos *et al.*, 1978).

MATERIALS AND METHODS

Preparation of polymorphonuclear leucocytes (PMN)
Human polymorphonuclear granulocytes were prepared from EDTA-blood of healthy blood donors by a technique modified from Bøyum (1968). The blood was sedimented on a mixture of 2/3 dextran (Pharmacia, Uppsala, Sweden) and 1/3 sodium metrizoate (Nyegaard, Oslo, Norway). The supernatant was subjected to hypotonic lysis and centrifuged (400 ***g***, 30 min) on Ficoll-Paque (Pharmacia) after restoration to isotonicity. The PMN-rich pellet was washed and resuspended to an appropriate concentration in Krebs-Ringer phosphate buffer containing 10 mM glucose (KRG), pH 7·2. The final PMN-preparation was only slightly contaminated with mononuclear leucocytes (< 1%) and erythrocytes (< 5%). Viability, when measured with trypan blue exclusion, was > 99%.

Yeast-particle preparations
Heat-killed yeast particles (*Saccharomyces cerevisiae*) were labelled with fluorescein isothiocyanate (FITC) (BBL Microbiology Systems, Cockeysville, MD) as previously described (Hed, 1977). The FITC-conjugated yeast particles (10^8 particles/ml) were then C3-opsonized in different concentrations of normal human serum (NHS) for 30 min at 37°. The serum-opsonized yeast particles, designated yeast-C3b in this study, have recently been characterized (Hed & Stendahl, 1982). After opsonization, the particles were washed and resuspended in KRG to be used in the immune adherence assay or in the phagocytic asssay. In some experiments the yeast particles were coated with rabbit anti-yeast IgG (14 μg/ml), as previously described (Hed & Stendahl, 1982), before opsonization in NHS. This peparation was designated yeast-IgG/C3b.

All preparations were counted and examined in a Bürker chamber immediately before use.

Immune adherence assay
Erythrocytes from human, rat or ox blood were mixed with an equal volume of differently treated yeast particles (erythrocytes/yeasts = 100/1) and incubated for 15 min at 37° with end-over-end rotation. In the inhibition studies, the suspensions were supplemented with 10 mM -mannose (Sigma Chemical Co., St Louis, MO) during the incubation period, or the erythrocytes were preincubated with monoclonal antibodies against the CR1 receptor (Gerdes *et al.*, 1982) (Dakopatts, Stockholm, Sweden). After incubation, two drops of the suspension were put on a glass side and examined under a Zeiss incident light microscope. The percentage of erythrocyte-attached yeast particles was calculated by counting a minimum of 100 yeast particles.

Phagocytic assay
A suspension of yeast particles (0·1 ml of 8×10^7 cells/ml) and erythrocytes (0·1 ml of 5×10^9 cells/ml) or erythrocyte lysate (from 5×10^8 cells/ml) or KRG in the control experiments were preincubated for 15 min at 37° with end-over-end rotation. After the preincubation, an equal volume of neutrophils (1×10^7 cells/ml), with or without catalase (2000 U/ml) (Sigma) and superoxide dismutase (200 U/ml) (Sigma), was added and the suspension was rotated for another 10 min at 37°. Immediately after the incubation period, one drop of the suspension and one drop of trypan blue (2 mg/ml) were put on a slide and examined at × 1250 magnification with a Zeiss incident light fluorescence microscope (Osram lamp HBO 50; filters 2xKp470, Lp455 and Lp520) by alternating between immunofluorescence and phase-contrast microscopy. The numbers of attached and ingested yeast particles were counted in 50 cells and could be differentiated by adding trypan blue, which quenches the extracellular fluorescence (FQ-method) (Hed, 1977).

Superoxide anion production
Superoxide-mediated cytochrome (cyt *c*) reduction was assayed essentially as described by Curnutte & Babior (1974). Ferricytochrome *c*, (0·1 ml, 15 mg/ml) (type VI, Sigma) was added to the samples in the final incubation with the target and the leucocytes, and incubated for 15 min at 37°. PMN kept at 4° were used as blanks. All samples were centrifuged for 10 min (1250 ***g***, 4°) and the supernatant was assayed for the amount of reduced cyt *c*, using a Beckman DU-6 spectrophotometer set at 550 nm. The amount of

reduced cyt *c* was calculated by using a millimolar extinction coefficient for cyt *c* of 15·5 at 550 nm.

Statistical analysis

Statistical significance of the results was analysed by Student's *t*-test or, where indicated, by the sign test for matched pairs.

RESULTS

Adherence of yeast-C3b to erythrocytes (immune adherence)

The adherence of yeast-C3b to erythrocyte CR1 receptors was examined by mixing yeast-C3b particles with increasing numbers of erythrocytes. The binding, expressed as the percentage of erythrocyte-bound yeast-C3b, increased with increasing number of erythrocytes added (Fig. 1). At the ratio 1:100, yeast-C3b: human erythrocytes, 74% of the yeast-C3b particles were bound, in contrast to an adherence of 15% when rat erythrocytes (lacking the CR1 receptor) were used. This ratio was selected for further experiments. The role of the CR1 receptor in the interaction was further studied by preincubating the erythrocytes with monoclonal anti-CR1, which decreased the adherence to 16%. An equally low percentage of interaction to human erythrocytes was obtained with IgG-opsonized (17·8%) and non-opsonized (12·9%) yeast particles. In order to test whether this low grade of adherence could be due to an interaction between yeast mannan and mannosyl-specific receptors on human erythrocytes, the experiments were performed with the addition of 10 mM L-mannose (Warr, 1980; Sung, Nelson & Silverstein, 1983) and with ox erythrocytes, known to lack such receptors (Duguid, Clegg & Wilson, 1979). The adherence was then further reduced (data not shown) to 7·0% in the former case and to 2·4% when ox erythrocytes were used.

The amount of C3b needed on the yeast surface to induce adherence was studied (Table 1) by varying the concentration of NHS opsonization (Hed & Stendahl, 1982). Binding was found to be negligible when less than 5% NHS was used. Opsonization in 5% NHS was used in further experiments, since we have recently shown that C3b-opsonization at high concentration causes a steric hindrance for anti-yeast IgG antibodies (Hed & Stendahl, 1984).

Phagocytosis of yeast-C3b

Yeast-C3b particles, preopsonized in 5% NHS, were incubated with PMN in the presence or absence of human erythrocytes. A small, but significant ($P<0·05$), increase (15%) in phagocytosis was observed when erythrocytes were present in the experi-

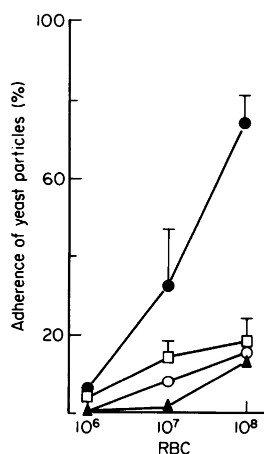

Figure 1. The adherence of yeast-C3b (●), yeast-IgG (□) and unopsonized yeast (▲) to human erythrocytes and the adherence of yeast-C3b to rat erythrocytes (○). 100 yeast particles were counted in each sample and the number of particles adhering to the erythrocytes at different concentrations was calculated ±SD ($n=10$).

Table 1. The adherence of yeast-C3b to human erythrocytes when opsonized in different concentrations of NHS

NHS (%)	% attached* ±SD	*n*	Significance
None	13±3	10	
			NS
1	16±5	6	
			***
5	43±4	6	
			**
25	50±7	6	
			***
50	74±7	10	

* 100 yeast particles were counted in each sample, and the number of particles adhering to the erythrocytes at the concentration 1×10^8/ml was calculated ±SD.

** $P<0·05$ (Student's *t*-test).

*** $P<0·001$ (Student's *t*-test).

ments (Fig. 2). A similar increase occurred upon the addition of catalase and superoxide dismutase, regardless of whether human erythrocytes were present or not. The ratio between intracellularly and extracellularly localized yeast particles was constant and no significant decrease in viability was observed. Reduced cytochrome *c* was measured in some experiments (Table 2), and a significant decrease was found when human erythrocytes were present. The level of reduced cytochrome *c* measured, however, was significantly lower than in the case of yeast-IgG/C3b, indicating that lower amounts of superoxide anion were released.

Phagocytosis of yeast-IgG/C3b

Yeast particles opsonized with both anti-yeast IgG and C3b (yeast-IgG/C3b) were used to investigate whether a scavenger effect of the erythrocytes would increase the phagocytosis of the target. No significant difference was seen in the immune adherence experiments with regard to the binding of yeast-C3b or yeast-IgG/C3b to erythrocytes. A 34% (Fig. 3a) increase in phagocytosis was obtained in systems with human erythrocytes using yeast-IgG/C3b as target, as compared to the 15% increase when yeast-C3b was used as the target (Fig. 2). The level of reduced cyt *c* was higher than in the previous experiments, as an expression of the greater metabolic response to yeast-IgG/C3b and a significant decrease of 42·8% was observed in the presence of human red blood cells during the phagocytosis (Table 2). Catalase and superoxide dismutase could replace the effect of human erythrocytes but did not display any synergy (Fig. 3a). Rat erythrocytes tended to decrease the phagocytosis (Fig. 3b). In contrast to its effect on the human system, the addition of scavengers when rat erythrocytes were present during the incubation increased the phagocytosis.

Erythrocyte lysate was used instead of whole cells to further test whether the increased phagocytosis was dependent on the CR1 receptor alone, or on a combination effect with erythrocyte-related scavengers (Lynch & Fridovich, 1978; Toth *et al.*, 1984). A 78%±19 ($n=9$) increase in phagocytosis was obtained when the lysate, corresponding to a 10% lysis of the erythrocyte concentration used in prior experiments, was added to the phagocytic system.

No difference in the ratio between intracellularly and extracellularly localized yeast particles was observed in the described experiments, nor was there any significant decrease of viability.

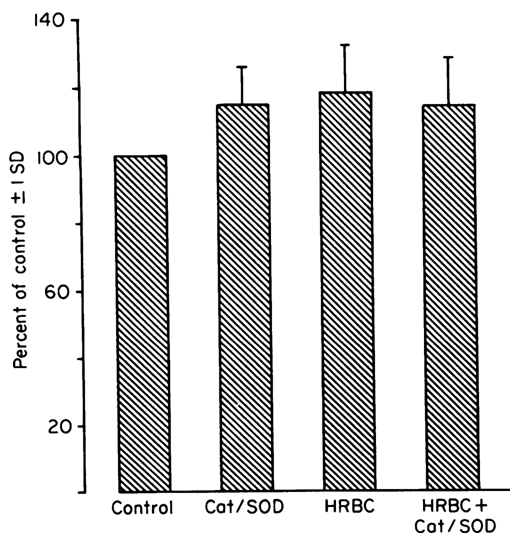

Figure 2. The total association of yeast-C3b to PMN in the presence or absence of human red blood cells (RBC) and/or catalase (Cat) and superoxide dismutase (SOD). The results are expressed as a percentage of the control (mean of nine experiments ±SD). The 100% value for the control was 78±3 particles/50 PMN.

DISCUSSION

Recent observations suggest that erythrocyte CR1 receptors have an important role in IC processing (Medof & Prince, 1983b); Medof *et al.*, 1982a). Approximately 95% of the CR1 receptors available in blood are located on erythrocytes (Siegel, Liu & Gleicher, 1981), and the majority of infused IC is rapidly bound to circulating erythrocytes (Cornacoff *et al.*, 1983). However, the exact role of erythrocytes in IC processing remains to be defined.

In this study, we have investigated whether erythrocytes would compete with neutrophils to bind complement-coated particles, as has been suggested in the case of IC (Medof & Oger, 1982b), or whether the phagocytic process would be enhanced.

We could show that the presence of erythrocytes increased phagocytosis of C3b-opsonized particles by 15% and IgG/C3b-opsonized particles by 34%. This increase in phagocytosis was only obtained with human erythrocytes and not with rat erythrocytes which are lacking CR1 receptors (Hajos *et al.*, 1978), thus indicating the importance of the binding of erythrocytes to the PMN. The larger increase obtained

Table 2. Superoxide anion release from PMN exposed to yeast, with or without human red blood cells (nmol of cyt *c* reduced/5 × 10^6 PMN)

Particle	−RBC*	+RBC*	% decrease*	*n*	Significance†
Yeast-C3b	10·5±5·2	7·4±2·4‡	25·9±5·1	5	$P<0·035$
Yeast-IgG/C3b	34·0±16·9	19·8±11·7§	43·8±7·0	5	$P<0·035$

* ±SD.
† Sign test of matched pairs.
‡ Adjusted by 1·15 due to increase of phagocytosis.
§ Adjusted by 1·34 due to increase of phagocytosis.

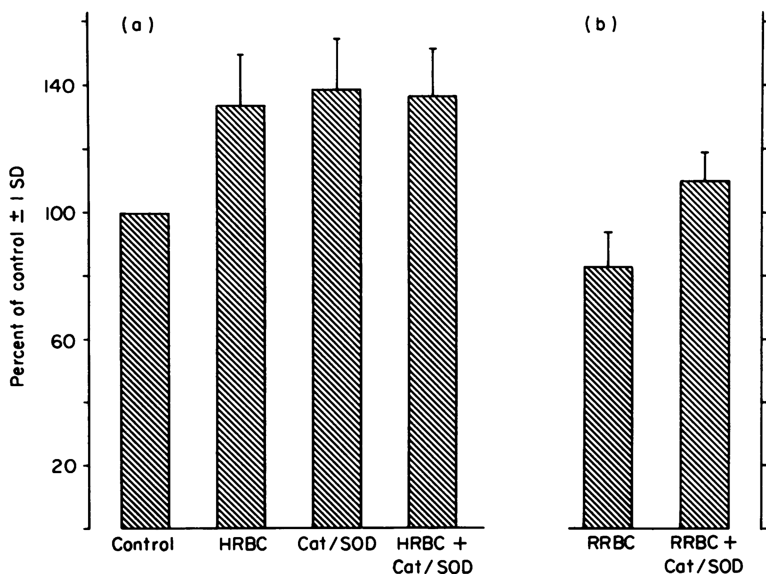

Figure 3. The total association of yeast-IgG/C3b to PMN in the presence or absence of human red blood cells [HRBC; (a)] or rat red blood cells [RRBC; (b)] and/or catalase (Cat) and superoxide dismutase (SOD). The results are expressed as a percentage of the control (mean of nine experiments±SD). The 100% value for the control was 84±12 particles/50 PMN.

with yeast-IgG/C3b particles was not due to increased adherence to human erythrocytes, since both types of particles interacted with erythrocytes to the same degree. The specifity of the CR1 binding was proven, as rat erythrocytes or monoclonal antibodies against the CR1 receptor abrogated the adherence between opsonized yeast and human red blood cells.

We have recently shown (Hed & Stendahl, 1982; Hed *et al.*, 1983), in agreement with results of others (Newman & Johnston, 1979; Johnston *et al.*, 1983), that the IgG bound to opsonized yeast particles is much more efficient than the bound C3b in inducing a metabolic response and a release of superoxide anion. Our experiments would also seem to support these observations, and we therefore studied this effect in more depth. We did this by measuring the amount of reduced cyt *c* as an expression of released, reactive superoxide anions, and also by adding scavengers, like catalase and SOD, to see whether the observed increase was due to a better presentation of the target to the phagocyte, or whether the erythrocytes' content of anti-oxidants could protect the neutrophils against the autotoxicity of released free radicals. The latter suggestion has already been made in another context

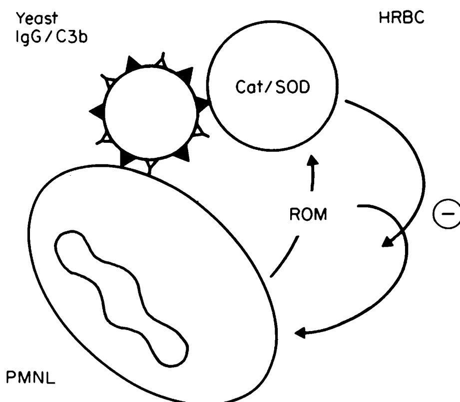

Figure 4. Concept of yeast-IgG/C3b interaction between human red blood cells and PMN during phagocytosis. See Discussion for details.

by Lynch & Fridovich (1978), and again recently by Toth *et al.* (1984). The erythrophagocytosis of red blood cells in patients with sickle cell anaemia was shown to suppress oxidative bactericidal mechanisms in macrophages, and it was suggested that this was due to interactions between erythrocyte components and ROM of the phagocyte (Hand & King-Thompson, 1983; Hand, 1984). Phagocytosis of yeast-C3b released small amounts of superoxide anions, which were further reduced when erythrocytes were included in the assay. Yeast-IgG/C3b released larger amounts of superoxide anions, and a decrease (by 43%) was also observed in the presence of human red blood cells, despite the increase of phagocytosis. Catalase and superoxide dismutase substitution augmented the phagocytosis of both yeast-C3b and yeast-IgG/C3b to the same extent as did the human erythrocytes: this result also indicates a primarily anti-oxidant role for the erythrocytes.

Human erythrocytes were lysed to see if the anti-oxidant content alone could, in fact, improve the function of neutrophils: a substantial increase of 78% was observed. This increase is larger than the increase observed when only catalase and SOD were used, and could be due to a more efficient release of other scavengers like glutathione peroxidase (Kellogg & Fridovich, 1977; Roos *et al.*, 1980) and haemoglobin (Hand, 1984).

Even though no significant erythrophagocytosis was observed, this does not exclude the possibility of such a mechanism being operative to a lesser extent. Superoxide anions are able to pass through the anion channels of the erythrocyte (Lynch & Fridovich, 1978), but erythrolysis may certainly also be a way to liberate scavengers. We have not yet studied how access to anti-oxidants comes about, but the results in this study suggest that the erythrocytes have to be very close to the PMN in order to enhance phagocytosis.

We have recently shown (Thaw *et al.*, 1984) that ROM modulates the IgG- and C3b-mediated phagocytosis of macrophages without affecting their viability. Present and recently published data thus suggest that erythrocytes enhance phagocytosis, rather than compete with the neutrophils for the target, and that this enhancement is due to the scavenging effect of the erythrocytes which are concurrently attached with the prey through its CR1 receptor.

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